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Epidemiological investigation of zoonotic infections in Hungary

Ph.D. Thesis

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ABBREVIATIONS

ADP	Adenosine Diphosphate
ALT	Alanine Aminotransferase
ALT	Alkaline Phosphatase
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
BiP	Binding Protein
BLAST	Basic Local Alignment Search Tool
CLIA	Chemiluminescence Immunoassay
CI	Confidence Interval
COVID-19	Ccoronavirus Disease of 2019
CRP	Cysteine-rich Protein
CTP	Cytidine Triphosphate
DNA	Deoxyribonucleic Acid
DTCS	Dye Terminator Cycle Sequencing
cDNA	complementary DNA
EB	Elementary Body
EIA	Enzyme Immunoassay
ELFA	Enzyme-Linked Fluorescent Assay
ELISA	**E**nzyme-Linked Immunosorbent Assay
ESCRT	Endosomal Sorting Complexes Required for Transport
FGR	Frequently Gene-Translocated Region
GGT	Gamma-Glutamyl Transpeptidase

Hel	HEV RNA helicase
HEV	Hepatitis E Virus
HIV	Human Immunodeficiency Virus
Hsp	Heat-Shock Protein
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFN-α	Interferon-Alpha
LPS	Lipopolysaccharide
MeT	Methyltransferase
MOMP	Major Outer Membrane Protein
NADH	Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H)
NAT	Nucleic Acid Test
Npt	Nucleoside Phosphate Transporter
NTPase	Nucleoside Triphosphatase
ORF	Open Reading Frame
PCP	Papain-Like Cysteine Protease
PCR	Polymerase Chain Reaction
POMP	Polymorphic Outer Membrane Protein
RB	Reticulate Body
RdRp	RNA-dependent RNA polymerase
RNA	**R**ibonucleic Acid
RR	Relative Risk
WHO	World Health Organization
qPCR	quantitative Polymerase Chain Reaction

(+)ssRNA positive-sense single-stranded RNA

(-)ssRNA negative-sense single-stranded RNA

1. INTRODUCTION

1.1. The growing importance of zoonotic infections

Most people interact with animals in different qualities. Zoonoses are infections that can spread naturally between vertebrate (non-human) animals and humans [1]. 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 [1,2]. These pathogens can spread to humans through direct contact with food, water, or environmental sources [2]. The emergence, re-emergence, spread, and pattern of zoonotic diseases are significantly influenced by climate change, urbanisation, animal migration and trade, travel and tourism, vector biology, and anthropogenic and natural factors. The prevalence of emerging and re-emerging zoonotic diseases has increased over time [1]. These zoonotic pathogens pose a significant public health challenge globally due to the close interactions between humans and animals in agriculture, as companions, and within natural environments. Additionally, zoonoses can disrupt the production and trade of animal products for food and other purposes. Zoonoses may represent a significant proportion of recently identified and existing infectious diseases. Certain pathogens, such as HIV, originated as zoonoses but now spread from human to human. Other zoonoses, like Ebola virus infections and salmonellosis, cause recurrent outbreaks, while coronaviruses, such as the novel coronavirus that was responsible for COVID-19, have the potential to cause global pandemics [2].

Some populations pose a higher risk for zoonotic diseases [3]. Individuals who work with animals or live near wilderness or semi-urban areas with higher populations of wild animals are at heightened risk [2,3]. 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 [2]. Within these groups, children, older people, and those with compromised immune systems are in an outstandingly vulnerable group [2,3]. Unfortunately, antimicrobial resistance complicates the management and prevention of zoonotic diseases. The widespread use of antibiotics in livestock increases the probability of developing drug-resistant strains of zoonotic pathogens, which can spread swiftly among animal and human populations [2].

Although our pets are great companions, they can also present significant human health risks. The number of pet animals has risen substantially over the past several decades, and they are a

notable reservoir of pathogens. Approximately 14-62% of pet owners permit their pets into their bedrooms, potentially facilitating the transmission of zoonotic diseases. The growing popularity of pets and companion animals has consequently increased the risk to human health due to the potential spread of infections [1].

Zoonotic diseases involve interaction between humans, animals, and the environment, requiring a multi-sectoral approach for effective control measures [1]. 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. Consequently, through the collaboration of different relevant fields, also known as the One Health approach, we are getting closer to overcoming the challenges caused by zoonoses [3]. The One Health concept has emerged as the international standard for zoonotic disease control in the past decade. This approach emphasises multisectoral collaboration among human, animal, and environmental health professionals. Despite an accurate definition or formal institutional governance, One Health has demonstrated its value. In a globalised world where human activities profoundly impact ecosystems indispensable to human and animal life, an interdisciplinary strategy is essential for achieving optimal health for people, animals, and the environment. The One Health approach integrates efforts across these domains and is now regarded as the global standard for combating epidemic zoonotic diseases [4].

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. The Introduction provides a comprehensive overview of all relevant aspects of the two pathogens, forming the basis of the thesis.

1.2. Detailed overview of hepatitis E virus

1.2.1. Taxonomic aspects

The hepatitis E virus (HEV) belongs to the *Orthohepevirus* genus in the *Hepeviridae* family, within the group of positive-sense single-stranded RNA ((+)ssRNA) viruses. The family includes two genera and five species. Within the *Orthohepevirus* genus, there are four species: *Orthohepevirus A* (HEV), *B*, *C*, and *D*. *Orthohepevirus A* mainly infect mammals (humans,

wild and domestic pigs, rats, rabbits, and deer) and molluscs, *Orthohepevirus B* infects birds, *Orthohepevirus C* infects rodents and *Orthohepevirus D* infects bats. The other genus is *Piscihepevirus*, where only one species, *Piscihepevirus A*, infects fish [5].

1.2.2. Structural and molecular characteristics

Hepatitis E virions are icosahedral and spherical particles without envelopes, with a diameter of about 27-34 nm. The virion capsid is built up by capsomeres composed of homodimers of a single capsid protein (Fig. 1). Each capsid protein (ORF2) consists of three domains: S, M, and P. Each domain contains a polysaccharide binding site that can interact with cellular receptors [6,7].

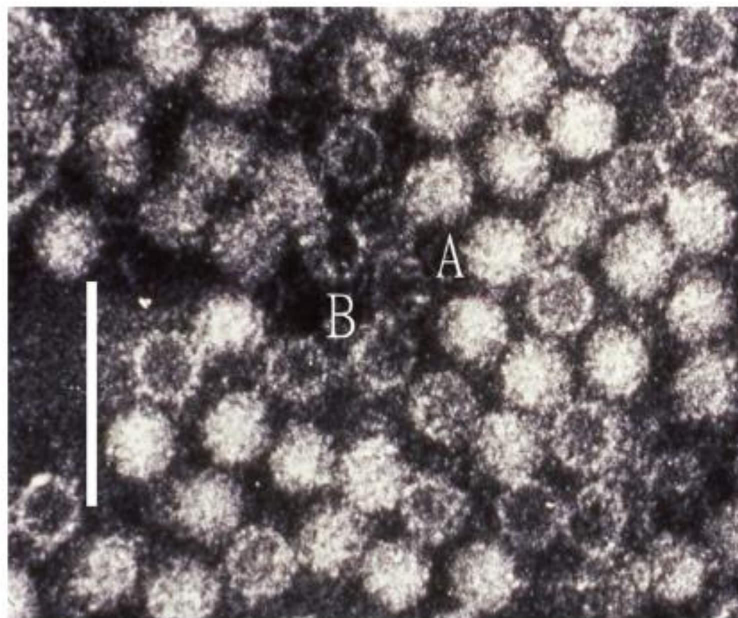

Figure 1. Electron micrograph of hepatitis E virions
(A) marks the virion, and (**B**) marks the empty capsid [7].

The HEV genome is a (+)ssRNA containing three open reading frames (ORF). ORF1 covers about two-thirds of the total length of the genome and encodes a protein called HEV replicase, which has several functional domains. These include methyltransferase (MeT), RNA helicase (Hel), RNA-dependent RNA polymerase (RdRp), and macrodomains (Fig. 2). The functions of the other domains, namely papain-like cysteine protease (PCP), variable (V), and Y domains, are not yet wholly characterised (Fig. 2). A new hypothesis suggests that the PCP domain can cleave ubiquitin from the target protein, thus the PCP domain may prevent proteasomal degradation of specific proteins, possibly enzymes required for RNA replication [8]. The MeT domain is responsible for RNA capping. The Hel domain is primarily involved in RNA duplex cleavage and exhibits 5'-nucleoside triphosphatase (NTPase) activity, which is required for RNA capping. Macrodomains function as ADP-ribose-binding modules that can interact with

ADP-ribosylation-modified proteins. This modification is essential in transcription, DNA repair, chromatin, and organelle organisation. ORF2 is located on subgenomic RNA synthesised by RdRp (Fig. 2) and encodes a capsid protein involved in virion assembly and interaction with target cells. The ORF2 protein can interact with various host factors throughout the viral life cycle. Examples of interacting factors are heat shock proteins (Hsp90), binding proteins BiP, or heparan sulfate proteoglycans. ORF3 is also located on subgenomic RNA (Fig. 2), overlapping with ORF2. It encodes a small protein that can interact with both the cytoskeleton and microtubules through its N-terminal hydrophobic domain. The ORF3 protein is responsible for virion morphogenesis and escape [8].

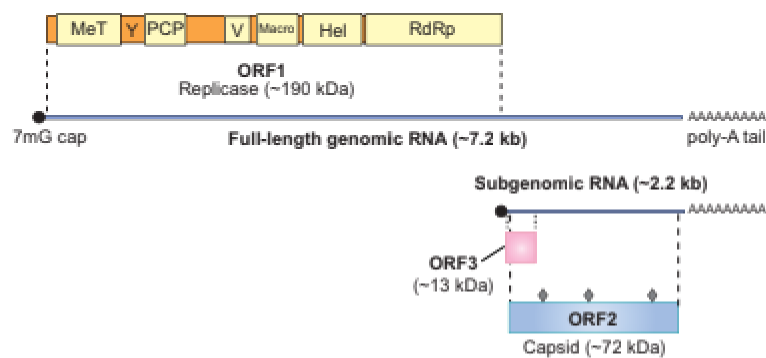

Figure 2. Structure of the HEV genome [8].

1.2.3. Genetic variability of HEV

To date, HEV can be phylogenetically divided into eight genotypes (HEV-1-8) [9]. The most prevalent of these are genotypes 1-4, major human pathogens. According to the currently accepted classification scheme, the four main genotypes of HEV can be further subclassified into subtypes, which have been defined based on 5' ORF1, 3' ORF1, 5' ORF2, 3' ORF2 and whole genome [10]. HEV-1 can be divided into five (1a-e) and HEV-2 into two (2a, 2b) subgenotypes. HEV-1 is mainly distributed in Asia, and HEV-2 is distributed in Africa and Mexico. Genotypes 1 and 2 have been associated exclusively with human infections and are prevalent mainly in developing countries [10]. HEV-3 is found throughout the world and is genetically quite diverse. Within the HEV-3 genotype, three monophyletic clades can be distinguished: group 1 (subtypes HEV-3e, f, and g), group 2 (HEV-3a, b, c, h, i, j, k, l, and m), and HEV-3ra (rabbit) [9]. HEV-4 is a very heterogeneous genotype, with at least nine subtypes (4a–4i), typically found in Southeast Asia and Central Europe [10,11]. Genotypes 3 and 4 can be carried by mammalian species other than humans (e.g. domestic pigs) [10]. HEV-5 and HEV-6 have been identified exclusively in wild boars in Japan. HEV-7 and HEV-8 have

recently been detected in camels in the Middle East and China. Among these four genotypes, only one human infection with HEV-7 has been reported, attributed to consuming contaminated camel meat and milk [9].

1.2.4. The life cycle of HEV

Viral infection, similar to other (+)ssRNA viruses, can be described by attachment to the host cell, penetration, RNA genome escape (decapitation), formation of early proteins, synthesis of complementary negative- and positive-sense ssRNA, formation of late proteins, assembly of viral particles, virion escape and finally host cell lysis (Fig. 3) [12].

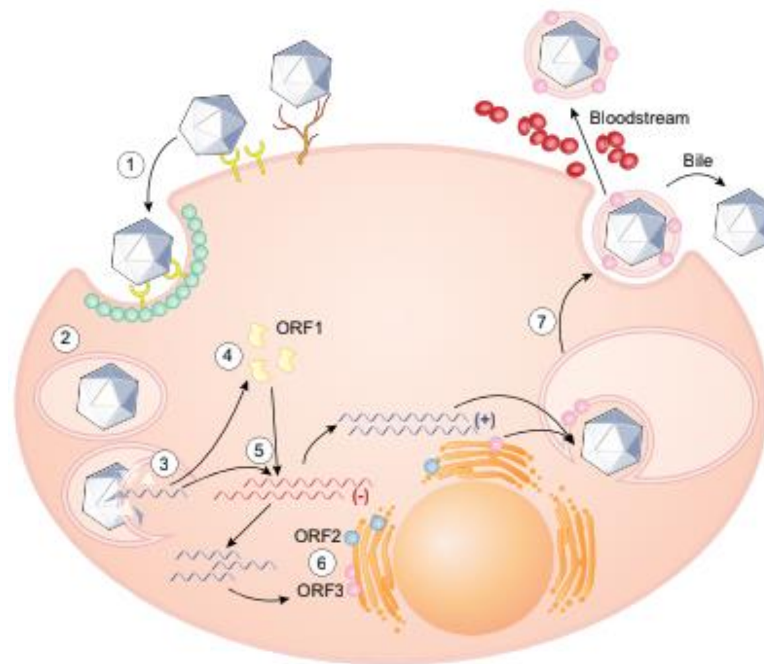

Figure 3. The HEV life cycle.
The steps in the life cycle are (**1**) viral attachment to heparin sulfate proteoglycans; (**2**) clathrin-dependent endocytosis; (**3**) RNA release (decapsidation); (**4**) translation of early proteins (ORF1); (**5**) synthesis of complementary negative-, then positive-sense genomic and subgenomic ssRNA; (**6**) synthesis of late proteins (ORF2, ORF3); (**7**) packaging, assembly and release [8].

Upon binding to the host cell, the HEV capsid proteins interact with heparan sulfate proteoglycans (syndecans). The virus enters the cell cytoplasm by clathrin-dependent endocytosis, where the (+)ssRNA genome is released from the viral particles. HEV replication first requires the synthesis of the viral replicase (ORF1) by the host cell translation system. RNA replication involves the synthesis of a negative complementary strand by RdRp, followed by synthesising genomic (+)ssRNA from the (-)ssRNA template. The RNA helicase performs the unwinding of the resulting RNA duplex and is involved in capping through the activity of NTPase. RdRp also synthesises subgenomic RNA. Subgenomic RNA, ORF2, and ORF3

proteins, and after that, the assembly of viral particles begins. ORF2 proteins (capsid proteins) are responsible for packaging genomic RNA. The virions are released from the host cell by the ORF3 protein, resulting in lysis of the cell. The ORF3 protein is most likely to bind to the intracellular membrane and trigger virion release via the ESCRT pathway. Density gradient centrifugation studies and structural analyses revealed that membranes of host cell origin envelop the released hepatitis E virions. Virions circulating in blood have envelopes, whereas virions in bile and faeces are envelope-less [8].

1.2.5. Clinical aspects of HEV infections

The clinical manifestations of HEV infection can range from asymptomatic infection to acute hepatitis or even chronic liver failure. A significant proportion of infections are asymptomatic [13]. The typical incubation period of HEV is approximately 2-8 weeks [10]. Primary symptoms may include jaundice, fever, joint and muscle pain, and abdominal pain. In some cases, headache, loss of appetite, weight loss, vomiting, nausea, intestinal complaints, and skin symptoms have also been observed. Symptoms usually resolve spontaneously in 4-6 weeks, as acute HEV infections are generally self-limiting. However, in some instances (e.g. pregnant women, patients with chronic liver disease, active alcohol users), acute liver failure may be more likely to develop. Chronic hepatitis E infection can lead to progressive liver fibrosis, irreversible liver cirrhosis (liver cirrhosis), and resulting liver failure, which may sometimes require liver transplantation [13].

HEV infection primarily can cause damage in the liver; however, with the detection of ssRNA in the kidney and spleen, it has been established that the virus can also replicate in other tissues [8]. Due to the faecal-oral transmission of HEV, the virus primarily replicates in the digestive system and can then reach other organs through the bloodstream [8]. Recently, numerous extrahepatic manifestations associated with hepatitis E infection have been reported. The most common are neurological manifestations, exclusively characteristic of HEV-1 and HEV-3 genotypes. The primary neurological diseases related to HEV include Guillain-Barré syndrome, brachial neuritis, neuralgia, acute myelitis, and acute meningoencephalitis. Other non-neurological extrahepatic manifestations have also been described, such as pancreatitis and various haematological disorders (aplastic anaemia, thrombocytopenia) [14].

1.2.6. Epidemiology of HEV infections in developing countries

Hepatitis E epidemics in developing countries are caused mainly by genotypes 1 and 2. HEV-1 is prevalent in Asia, and HEV-2 is commonplace in Africa and Mexico (Fig. 4) [10]. A retrospective study confirmed that 29,300 people were infected during India's 1955-56 HEV

epidemic [15]. Similar hepatitis E epidemics affecting thousands of people have been reported in China, Somalia, and Uganda [15]. In addition to outbreaks, sporadic cases also occur in endemic areas [10]. Viral infection is mainly transmitted through water with faecal contamination (Fig. 5). Human-to-human transmission is sporadic. Still, studies in Uganda suggest that household and hygiene conditions (e.g. lack of hand hygiene, shared hand washing utensils and plates) may also play a role in hepatitis E outbreaks [10,15]. Mortality associated with outbreaks is typically 0.2-4.0%. For unknown reasons, the mortality rate is relatively high in infants under two years of age and 10-25% in pregnant women. Maternal deaths occur primarily in the third trimester, caused mainly by liver failure or obstetric complications (eclampsia, haemorrhage) [15]. Outbreaks of waterborne hepatitis E mainly affect young adults (typically 15-35 years old) [6]. The disease is much more common among men in developing countries. Up to two to five times more men than women can be infected with the virus. Asymptomatic cases can be up to 2-4 times higher than symptomatic infections in waterborne hepatitis E epidemics [15]. China is typically classified as an HEV-endemic area. Most infections are associated with HEV-1 and HEV-4 genotypes (Fig. 4), although HEV-3 has also been detected within the region. HEV-4 cases are generally the most common, but the 1989 outbreak in Xinjiang province was caused by HEV-1 and affected about 120,000 people. Sporadic cases associated with HEV-4 are more common among older men, similar to HEV-3. In China, the reasons for the spread of HEV-4 are not yet well understood, but improvements in health care, water supply, and infrastructure have likely led to the recent dominance of the zoonotic HEV-4 genotype [15].

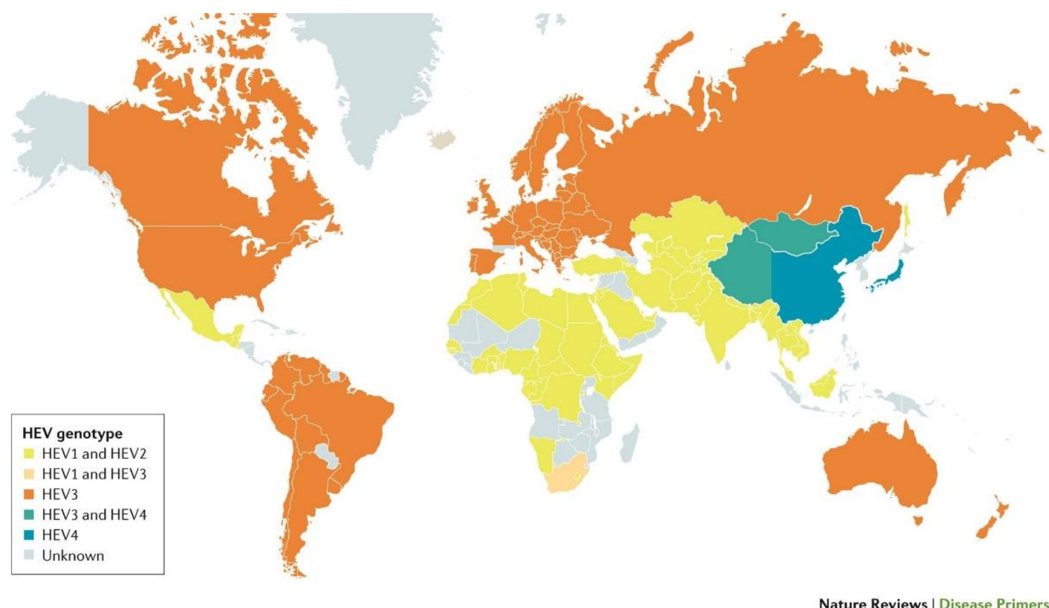

Figure 4. Prevalence of HEV infection worldwide [16].

1.2.7. Epidemiology of HEV infections in developed countries

Recently, it has become clear that HEV is endemic in many developed countries. Several studies have shown that autochthonous hepatitis E infection is a severe problem in Japan, New Zealand, Europe, and North America (Fig. 4) [6]. In contrast to developing countries, hepatitis E infections are mostly zoonotic diseases caused by the HEV-3 and HEV-4 genotypes, which are mainly spread through the consumption of inadequately heat-treated pork and game meat (Fig. 5) [6,17]. HEV infection in developed regions primarily affects middle-aged and older men [6]. According to several international studies, hepatitis E infection is more common among excessive ethanol consumers. This may be because alcohol consumption increases the risk of developing liver diseases (e.g. liver fibrosis), which may contribute to a more severe manifestation of HEV infection [6,15]. This is supported by a UK study in which 6 (13%) of 47 patients with drug-induced liver failure were found to have a pathogenic role for HEV-3 [15]. Studies from the UK confirm that HEV infection is also common in coastal areas and is associated with the consumption of shellfish and crabs (Fig. 5) [15].

In developed countries, seroprevalence data show a wide range (11-52%) [15]. International studies confirmed that the incidence of hepatitis E infection has increased in many European countries over the last ten years [18-20]. In Europe, 5-15% of acute hepatitis of unknown origin is associated with HEV infection. However, the seroprevalence varies from country to country. In addition to this, regional differences in some countries could be observed. For example, in France, seroprevalences range from 8 to 86.4%, and in Spain, values vary from 0.8 to 7.3% [21]. A study conducted in Hungary from 2001 to 2006 established that HEV is an endemic agent in the country. The study found that HEV caused nearly 10% of unknown hepatitis cases in humans, and for the first time in the country, HEV was detected in domestic animals (pigs) and wild animals (roe-deer, wild boar). According to the study, the seroprevalence in Hungary was 18.4% [22].

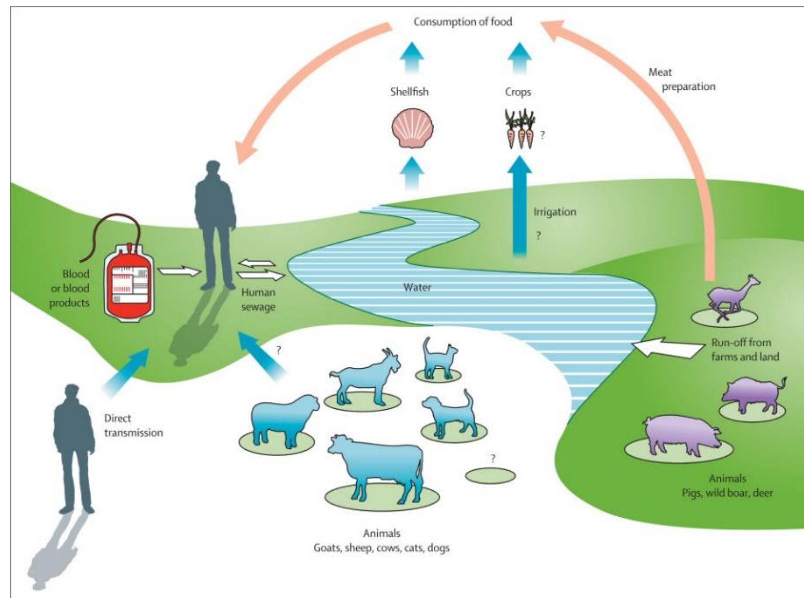

Figure 5. Sources of HEV-1-4 infection [15].

1.2.8. Zoonotic reservoirs of HEV in developed regions

In developed regions, hepatitis E infections are mainly associated with the zoonotic genotypes HEV-3 and HEV-4. Several mammalian species (e.g. deer, rabbits, wild and domestic pigs) can carry the virus. Still, domestic pigs are considered the primary host of HEV (Fig. 5). HEV is apathogenic to pigs and can be detected in pig populations worldwide [6,17]. In Japan, 2% of pig livers intended for human consumption tested positive for HEV RNA. This proportion was 11% in the USA [23]. HEV heat stability studies have shown that the virus can remain viable for up to 1 hour at 56°C and requires cooking at 71°C for 20 minutes to achieve complete inactivation. Another possible way of transmitting HEV infection is through direct contact with infected animals. Seroprevalence studies have demonstrated a higher rate of seropositivity among veterinarians and pig farmers than in the general population [6,23].

1.2.9. Characteristics of chronic hepatitis E

Previous studies have shown that hepatitis E infection is primarily a self-limiting disease; however, based on data from recent years, chronic infection is becoming more common [10]. Chronic hepatitis E infection is defined as HEV replication that persists for at least six months [6]. Chronic infection has been confirmed primarily in transplanted patients, but chronic disease has also been reported in HIV-infected patients, patients treated with chemotherapy, and immunosuppressed patients [6,10]. Viral transmission with transplanted organs has also been reported, and therefore, such infection may occur in this patient group [6]. Similar to the seroprevalence data for the general population, the seroprevalence among transplant patients is

2.3-43.9%. In adult transplant recipients, chronic infection may develop in about 60% of HEV-infected patients, while cirrhosis may be observed in 10% of chronically infected patients within a short time (3-5 years) [14]. Overall, the incidence of infection, based on HEV RNA detection, is approximately 0.9-3.5% in immunosuppressed individuals [6].

1.2.10. Laboratory diagnosis of HEV infection

HEV infection is difficult to distinguish from other types of acute viral hepatitis based on clinical signs and symptoms, so laboratory tests can be used to make the diagnosis. The laboratory tests are partly based on the determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), bilirubin and alkaline phosphatase levels (ALP). AST and ALT levels are the main indicators of liver function following HEV infection. In case of suspected HEV infection, serum/plasma and faecal samples are suitable for microbiological testing. Detection of HEV can be indirect (serological), i.e. by detection of the immune response (ELISA), or direct (molecular), i.e. by detection of HEV RNA (PCR) [10,13].

1.2.10.1. Serological tests

Anti-HEV IgM is detected in the acute stage of infection (usually four days after the onset of jaundice) and may be detectable for up to 5 months. Still, strong positive reactions are rare even after three months. Anti-HEV IgM antibodies are detectable in 90% of patients after two weeks of the onset of infection, and IgG is detectable shortly after the IgM response (Fig. 6). The fact that both types of antibodies are present in the blood simultaneously during acute infection makes accurate diagnosis quite difficult [10]. The commercially available ELISA, ELFA, and CLIA tests can differ significantly in sensitivity and specificity [10,14]. The only presence of anti-HEV IgG may indicate previous hepatitis E infection. In IgM-positive cases, acute HEV infection can be confirmed by seroconversion. Due to the prolonged persistence of HEV-specific IgM, acute infection can be confirmed by IgG avidity testing or RNA detection [6].

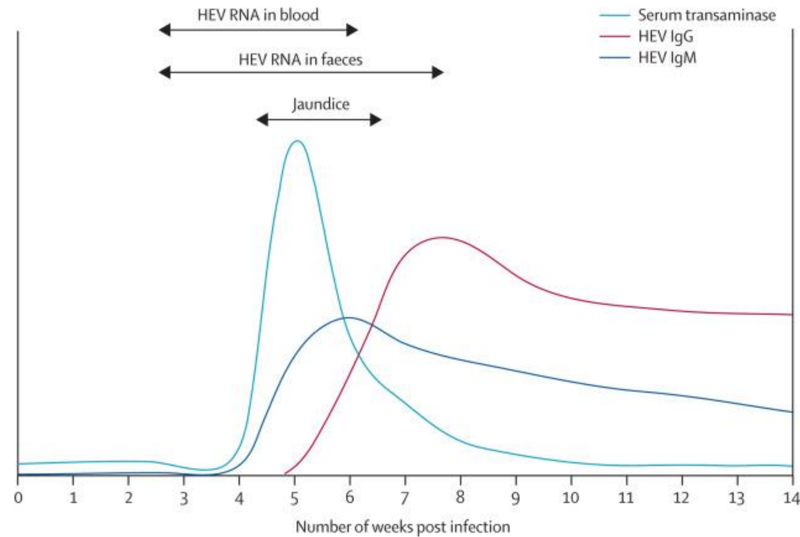

Figure 6. Markers are used in laboratory diagnosis of HEV infections [15].

1.2.10.2. Molecular detection of HEV

The detection of HEV RNA is critical in diagnosing hepatitis E infections [15]. The sensitivity of HEV RNA detection is highly dependent on the timely collection of serum and stool samples and proper transport and processing. Even though RNA can be detected in serum and faeces for up to 4-6 weeks, the amount of RNA can be very low, reducing the detection success rate [10]. Several studies have used nucleic acid-based tests (NATs) for HEV RNA detection in the last few years. Such methods include real-time PCR, reverse transcription PCR, or nested PCR. The majority of NATs are suitable for the detection of all four human HEV genotypes [10]. HEV RNA assays are crucial in immunosuppressed patients due to their impaired immune responses and the lower reliability of IgM tests in this population. HEV RNA detection is important for molecular characterisation for these patients and is essential to identify chronic infection. If HEV RNA is detectable over three months, it indicates a low probability of spontaneous viral clearance without therapeutic intervention [6].

1.2.11. Preventing infection

HEV infection can be reduced by minimising the possibility of contracting the virus, e.g., by improving hygiene and using a vaccine. The main prevention strategy for developing countries focuses on improving health infrastructure and providing clean drinking water. The situation is more complex for developed countries, as there are many possible routes of HEV infection. For example, proper preparation of meat products and screening of donors and blood products for HEV in the case of transplantation can be an effective form of prevention [15]. The best-known vaccine to date is Hecolin (HEV 239), a 26 kDa recombinant protein encoded

by ORF2 of HEV-1 and expressed in *Escherichia coli*. Studies in China have shown that the vaccine is safe and immunogenic. It has been approved for use in China. However, more data are needed to ensure its wider uptake [6,24].

1.2.12. Therapeutic possibilities in the case of HEV infections

Because of the self-limiting nature of acute HEV infection, it does not require treatment in most cases. If severe liver failure develops, ribavirin can be used as monotherapy, or ribavirin can be combined with sofosbuvir, which inhibits the replication of HEV-3 RNA. The use of ribavirin in pregnant women with HEV infection is not recommended because of its teratogenic effects. However, the infection may carry significant risks for both the mother and the fetus, therefore, trials of antiviral therapy might be worthwhile [15]. Antiviral therapy should be considered in immunosuppressed patients due to the increased risk of chronic HEV infection. Reduction of the dose of immunosuppressive agents, combined with IFN-α (interferon alpha) and ribavirin, may address the problem [13]. Some reports suggest that reducing the tacrolimus dose may be successful in 30% of transplant patients [13]. Many immunosuppressive drugs can inhibit the T-cell immune response. This may be one reason why transplant patients can easily develop chronic hepatitis E from acute infection. To avoid this in HIV-infected patients, antiretroviral therapy is optimised to increase CD4+ (T helper cell) cell counts, which prevents the infection from becoming chronic [13]. Antiviral treatment is recommended for patients who cannot reduce immunosuppressive treatment due to transplantation or other reasons or cannot achieve viral clearance despite antiviral treatment [13].

1.3. Detailed overview of *Chlamydia felis*

1.3.1. Taxonomic details and zoonotic aspects

The phylum *Chlamydiae* is a group of obligate intracellular bacteria commonly found in the environment and infect humans and various animals, including amoebae, insects, aquatic animals, reptiles, birds, and mammals [25]. The *Chlamydiaceae* family is the most significant pathogenic group within this phylum, which contains two genera: *Chlamydia* and *Chlamydiifater*. The genus *Chlamydia* includes the following identified species: *C. abortus, C. avium, C. buteonis, C. caviae, C. crocodili, C. felis, C. gallinacea, C. muridarum, C. pecorum, C. pneumoniae, C. poikilothermis, C. psittaci, C. serpentis, C. suis, and C. trachomatis*. Additionally, there are four Candidatus species: *Ca. Chlamydia corallus, Ca. Chlamydia ibidis, Ca. Chlamydia sanzinia, and Ca. Chlamydia testudines. Chlamydiifrater*, a newly described genus, includes two recently identified species: *C. phoenicopteri* and *C. volucris* [25,26].

Within the *Chlamydia* genus, in addition to the strictly human pathogen *Chlamydia trachomatis*, various animal-associated species can infect vertebrate animals, particularly mammals, birds, and reptiles [25,27,28]. Inside the genus are important economic pathogens such as *C. suis, C. abortus, C. pecorum, and C. psittaci.* These pathogens can primarily infect livestock hosts (pigs, sheep, cattle, horses, and poultry) and cause severe diseases, resulting in significant losses [25]. Chlamydiosis in these animals can cause a broad range of diseases, such as atypical pneumonia, enteritis, conjunctivitis, endocarditis, and even abortion, mainly due to *C. abortus* and *C. psittaci* infections [27,29].

Several *Chlamydia* species are transmissible to humans and pose serious public health challenges, potentially causing pneumonia, atherosclerosis, coronary heart disease, and other severe illnesses. The diseases caused by these microorganisms warrant increased attention due to their zoonotic aspects and the possible zoonotic potential of other animal pathogens. [27]. *C. felis* and *C. caviae* primarily infect household pets like cats and guinea pigs. Moreover, these infected pets can transmit the infection to their owners [25,28]. Although *C. pneumoniae* is primarily a common respiratory pathogen in humans, it can also infect many animals, including horses, cattle, cats, dogs, various reptiles, and amphibians [28,29]. In addition to the previously mentioned species, *C. felis* is prominent among those with zoonotic potential. This pathogen most commonly infects cats' upper respiratory tract and eyes, causing respiratory symptoms and conjunctivitis. Infected cats spread the pathogen through secretions from these areas. According to the literature, dogs can also become infected, presenting clinical symptoms similar to those observed in cats [27]. *C. felis* is not only associated with mammals. It has also been reported in reptiles, such as iguanas [29]. Although C. *felis* is the main *Chlamydia* species associated with cats, other chlamydial species, particularly *C. abortus*, *C. pneumoniae*, *C. psittaci*, *C. suis*, and *C. caviae* have occasionally been reported as well [30-34]. Moreover, symptoms such as conjunctivitis in cats and dogs caused by other *Chlamydia* species, more precisely *C. psittaci* and *C. pneumoniae,* have been reported [32,34-36]. Both dogs and domestic cats are increasingly common household pets and faithful friends and companions of humans; however, it is crucial to be aware that these animals, primarily cats, may be significant sources of chlamydial infections [27,37].

1.3.2. Biology of *C. felis*

The genome of *C. felis* is small and has been sequenced based on a previous study (Fig. 7) [36,38]. The *C. felis* genome comprises a circular 1,166,239 bp chromosome harbouring 1,005 protein-coding genes and a 7,552 bp circular plasmid [38]. A former study has suggested

that the cryptic plasmid is necessary for pathogenicity, though the detailed mechanism is unknown [39]. A comparison of *C. felis* gene contents with other *Chlamydia* species shows that 795 genes are common in the family *Chlamydiaceae* species, and 47 genes are specific to *C. felis*. Phylogenetic analysis of these shared genes shows that most orthologous gene sets have similar divergence patterns. However, 14 genes in *C. felis* have accumulated more mutations, suggesting these genes may be involved in evolutionary adaptation to the specific niche of *C. felis*. Gene distribution and orthologue analyses indicate that two distinct regions, the plasticity zone and frequently gene-translocated regions (FGRs), likely play significant but different roles in the evolution of the chlamydial genome [38].

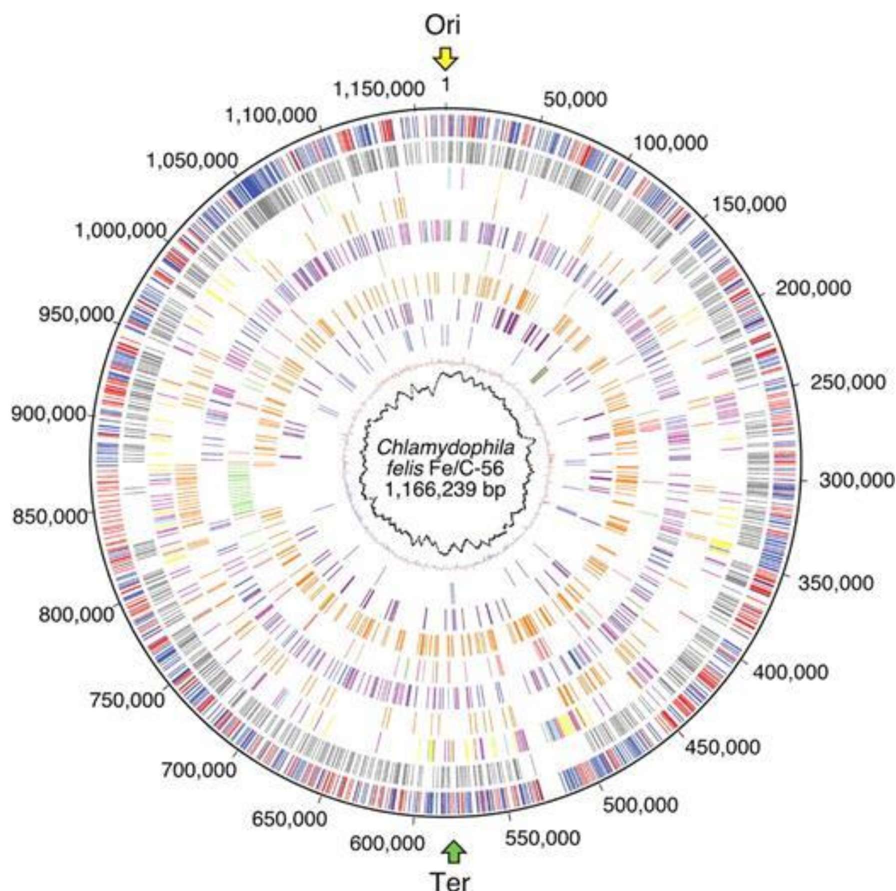

Figure 7. *C. felis* genome of a Japanese *C. felis* strain Fe/C-56, sequenced in a study by Azuma *et al.* [38].

C. felis is a rod-shaped coccoid bacterium with a cell wall similar to the Gram-negative bacteria, having a high lipid content but lacking peptidoglycan (Fig. 8) [36,40]. The membrane contains essential families of proteins: major outer membrane proteins (MOMPs) and polymorphic outer membrane proteins (POMPs) [39]. The outer membrane consists of lipopolysaccharides (LPS) and genus-specific heat-shock proteins (Hsp) (Fig. 8) [40]. Genetic analysis of the outer

membrane protein genes of *C. felis* indicates that all feline isolates are genetically similar. However, serological methods and DNA fingerprinting suggest the existence of more than one strain of *C. felis* [36].

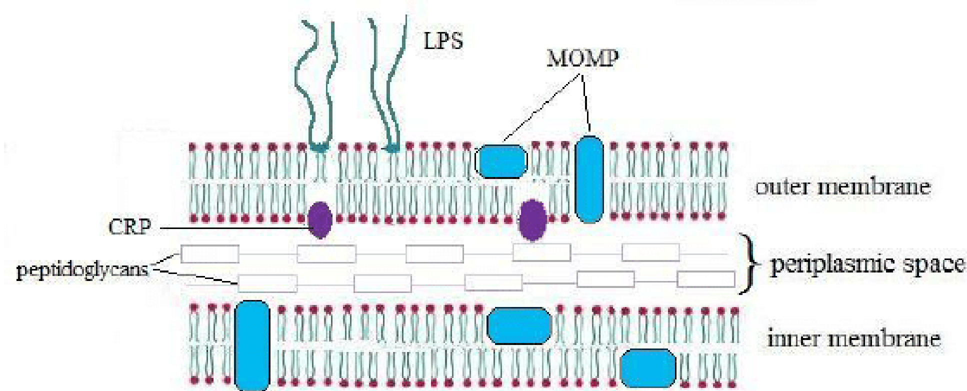

Figure 8. Structure of the *Chlamydia* cell wall. (CRP) cysteine-rich protein; (LPS) lipopolysaccharide; (MOMP) major outer membrane protein [40].

1.3.3. Metabolism of *C. felis*

For many years, bacteria from the family *Chlamydiaceae* were regarded as strict "energy parasites," dependent on the host's adenosine triphosphate (ATP) to meet their energy requirements. This belief originated from early biochemical results which showed that *Chlamydia* species lacked detectable succinoxidase and cytochrome c reductase activities, indicating the absence of several essential components of the mitochondrial respiratory chain, such as flavoproteins and cytochromes [41,42]. Later discoveries revealed that *Chlamydia* species possess two nucleotide transporters, Npt1 and Npt2, enabling them to import nucleotides from their host. This, coupled with the finding that *Chlamydiaceae* cannot synthesise nucleotides *de novo*, except for cytidine triphosphate (CTP), further supported the energy parasite concept [41,43,44].

C. felis can utilise the host cell's nutrients and perform glycolysis and the citric acid cycle. Additionally, the bacteria possess an operational respiratory chain that includes components such as a Na+-translocating NADH dehydrogenase, succinate dehydrogenase, cytochrome bd oxidase, and a vacuolar-type adenosine triphosphatase (V-type ATPase). A cytochrome bd-like oxidase, usually exhibiting high oxygen affinity, was proposed to indicate that *Chlamydiae* may face reduced oxygen conditions during intracellular growth and imply a microaerophilic lifestyle. The existence of a Na+-translocating NADH dehydrogenase suggests that the bacteria generate an electrochemical gradient across the plasma membrane using a sodium-motive force. Furthermore, *C. felis* is equipped with complete pentose phosphate and gluconeogenesis pathways, capable of synthesising and degrading glycogen [45].

1.3.4. The chlamydial developmental cycle

All *Chlamydiae* share a similar developmental cycle, transitioning between two primary cell types: the elementary body (EB), which is an infectious, non-dividing extracellular form, and the reticulate body (RB), which is an intracellular replicative form. RBs are morphologically similar among all members of the *Chlamydiae*, while EBs vary significantly in size and shape [45]. Infection begins with the attachment to sialic acid receptors of host cells and internalisation (endocytosis) of an EB, which remains within a non-fusogenic vesicle known as the inclusion [36,45]. Once the EB begins its developmental cycle, it quickly loses its ability to cause infection, signalling the start of its transformation into an RB. During the first 10 to 12 hours of this process, EBs increase in size as they differentiate into RBs and prepare for replication. Around 18 hours into the cycle, the RBs multiply, while a subset begins to revert to EBs asynchronously. These EBs accumulate within the inclusion until they are eventually released through cell lysis or extrusion, typically around 48 hours or later. After all this, released EBs can infect further host cells (Fig. 9) [45].

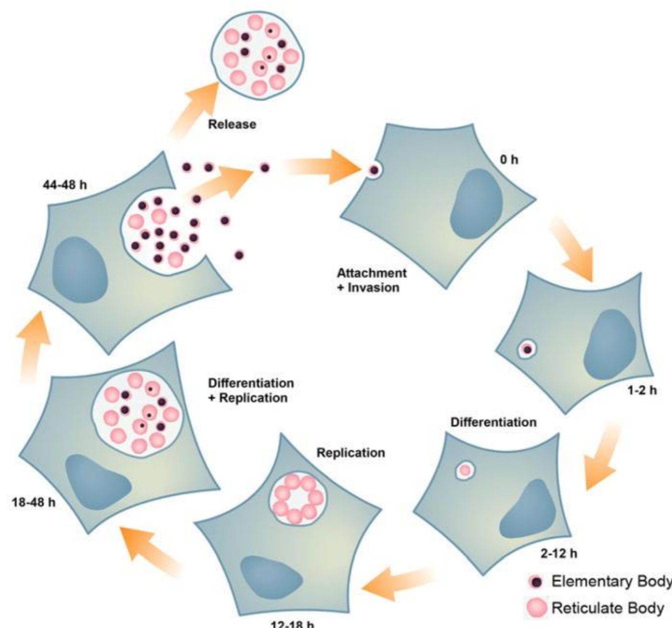

Figure 9. Chlamydial life cycle [45].

1.3.5. Clinical features

Chlamydia species primarily target mucosal tissues, and the main target for *C. felis* is the conjunctiva [27,36]. After 2 to 7 days of incubation, infected cats typically manifest conjunctivitis (Fig. 10) [30,46]. This condition often begins as unilateral but commonly progresses to involve both eyes. It is generally marked by conjunctival chemosis, blepharospasm, ocular discharge, excessive tearing, and hyperemia of the nictitating membrane. The ocular discharge is initially serous but may evolve to a more mucoid or

mucopurulent consistency [30,47]. Additionally, some cats may exhibit systemic signs such as fever, lethargy, sneezing, serous nasal discharge, submandibular lymph node enlargement, lameness, inappetence, and, in kittens, impaired weight gain [30,46]. Although some cats may recover more quickly, most untreated cases develop chronic conjunctivitis, with ocular symptoms typically persisting for 22 to 45 days. Ocular shedding of the pathogen generally stops around 60 days post-infection [30]. However, *Chlamydiae* has been detected in conjunctival samples of experimentally infected cats up to 8 months post-infection, indicating that some cats may remain persistently infected as asymptomatic carriers over an extended period [30,48]. *C. felis* can also spread from the eye via the bloodstream to other organs, including the tonsils, lungs, liver, spleen, gastrointestinal tract, and kidneys [36]. The clinical manifestations in dogs are analogous to those observed in canine distemper and include conditions such as conjunctivitis, encephalitis, pneumonia, and keratitis [27].

Kittens initially receive protection against chlamydial infection through maternally derived antibodies, which offer passive immunity during the first one to two months of life [36]. The mechanisms underlying active immunity to chlamydial infection in cats are not fully understood, though cellular immune responses are believed to play a critical role [49]. MOMPs and POMPs have been implicated in various aspects of chlamydial infection and disease, such as the induction of host immune responses [50]. However, immunity following natural infection appears short-lived and does not prevent reinfection. In addition, there may be an age-related resistance to infection [36].

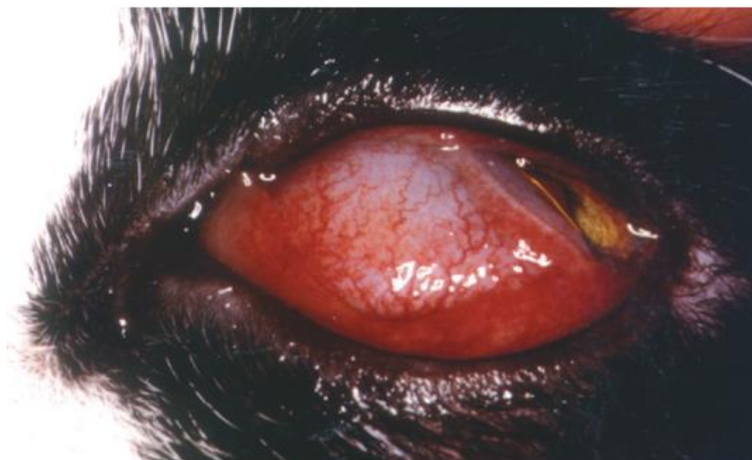

Figure 10. Conjunctivitis caused by *Chlamydia felis* in a cat [36].

1.3.6. Epidemiological characteristics of *C. felis* infections

Natural transmission of *C. felis* requires close contact between infected cats and their aerosols or fomites with ocular secretions, considered the most critical infection source [30]. A

previous study reported that experimental ocular infection in cats resulted in chlamydial shedding from the vagina and rectum in 50% and 40% of cases, respectively, indicating that *C. felis* is not confined to the conjunctival mucosa. The study proposed that primary ocular chlamydiosis may result in persistent infection of the genital and gastrointestinal tract [30,48]. Subsequent studies have confirmed the presence of *C. felis* in the reproductive tracts of both experimentally and naturally infected cats. However, the potential for venereal transmission and its epidemiological significance remain unclear. These findings support the hypothesis that the faecal-oral route might be an alternative infection pathway for *C. felis* [30,48,51]. It has been reported that chlamydial infections are usually higher during the spring and summer months. Additionally, it has been observed that the prevalence of infection is significantly higher in cats younger than one year compared to those older than one year. Therefore, there may be an age-related inclination and seasonality in *C. felis* infections among cats [30,52].

Since *C. felis* transmission requires close contact between cats because of its low viability outside the host, thus transmission by ocular or conjunctival secretions occurs primarily in cats living near each other [36]. Therefore, infections are common in breeding catteries and shelters and among pedigree cats [30,36]. Studies have reported a high prevalence of *C. felis* in shelters and stray cats, including those with conjunctivitis. Still, it generally poses a lesser threat compared to respiratory viruses [53,54,55]. According to several recent studies from different countries (using PCR, DNA microarray, isolation, or immunofluorescence assays), the chlamydial prevalence in pet cats ranges from 0% to 10% in asymptomatic animals and 5.6% to 30.9% in cats with conjunctivitis. In stray cat populations, the prevalence is typically higher, with overall positivity rates of 24.4% to 35.7%, but can reach up to 65.8% in subgroups with conjunctivitis [30]. In the most recent studies in China, the positivity rate was 11.76% in symptomatic stray cats (higher in Jiading District: 23.53%) and 11.62% in symptomatic domestic cats [56,57]. No epidemiological data (from animal or human health perspectives) on the prevalence of *C. felis* infections are available in Hungary.

C. felis is not often recognised as a disease-causing pathogen in dogs. However, some studies have identified *C. felis* infections in asymptomatic dogs by real-time PCR assays. Pantchev *et al.* reported that *C. felis* is particularly well adapted to cats. Although the positivity rate in dogs was high in that study, dogs infected with *C. felis* rarely developed conjunctivitis, unlike cats [27,33,58]. In seroprevalence studies conducted in China, antibodies to *C. felis* were found in 32 (12.1%) of 264 pet dogs, which was higher than the previously reported result (2.87%) in Dongguan, southern China [27,59].

Zoonotic transmissions of *C. felis* in humans are rarely reported, except for some cases, demonstrating the potential risk for transmission from infected animals to humans [28]. Some previous studies have reported conjunctivitis caused by *C. felis* in humans [60-62]. A novel research summarising three cases reported that in three patients with symptoms of conjunctivitis, living in close contact with their cats, *C. felis* was detected. The findings highlight that zoonotic infections in atypical conjunctivitis require specific PCR testing for diagnosis and show that instead of azithromycin, doxycycline is more effective for treatment, as in the case of cats. All three patients have made a complete, symptom-free recovery [63].

1.3.7. Diagnostic possibilities in the case of chlamydiosis

Culture techniques can detect chlamydial infections. However, this method has largely been replaced by more sensitive molecular diagnostics, such as PCR. As a result, culture is primarily reserved for research purposes. The culture process necessitates specialised chlamydial transport media to preserve the viability of the conjunctival swab samples during transportation. False-negative results can occur if the chlamydial organisms lose viability during transport or the infection is chronic, resulting in a low organism copy number [36]. PCR technique has become the "gold standard" for diagnosing chlamydial infections due to their high sensitivity and specificity and avoiding issues related to the poor viability of the organisms. Quantitative PCR (qPCR) methods are also available, allowing for precise quantification of the pathogen load [36,64]. While ocular/conjunctival swabs are primarily used for sampling, studies have shown no significant difference in the detection of *C. felis* via PCR across different types of swabs, including oropharyngeal, nasal, and buccal swabs [36,65,66]. Although organisms can be detected in vaginal swabs, aborted fetuses, and rectal swabs, these sample types are rarely used for diagnostic purposes. Given that the organism is intracellular, obtaining high-quality swabs that capture the infected epithelial cells is essential to ensure accurate detection [36]. The most successful collection devices are cytology brushes, as they can collect more cells, but flocked swabs have also proven effective [30]. Both genus- and species-specific PCR assays are suitable for detecting *C. felis*. Alternatively, sequencing of amplified PCR products can be applicable to identify the pathogen at the species level [30,36]. Furthermore, a recombinase-aided amplification assay is also developed to detect *C. felis*, but it is not widely known [36,67].

As an additional method, conjunctival swabs can be Giemsa-stained to identify cytoplasmic inclusions. However, these chlamydial inclusions are easily mistaken for other basophilic inclusions, such as melanin granules. They are typically only present in the early stages of

infection, resulting in low sensitivity [36]. Immunofluorescence and ELISA techniques are used for quantitative or qualitative measurement of antibody titers (Fig. 11). Serology can be helpful to establish whether infection is endemic in a group and can be valuable to diagnosing previous or current *C. felis* infections in unvaccinated cats. Vaccination with a chlamydial vaccine may interfere with result interpretation, and cross-reactivity with other bacterial species can also occur [36]. Specific ELISA tests can distinguish naturally *C. felis*-infected cats from vaccinated and non-infected cats [68].

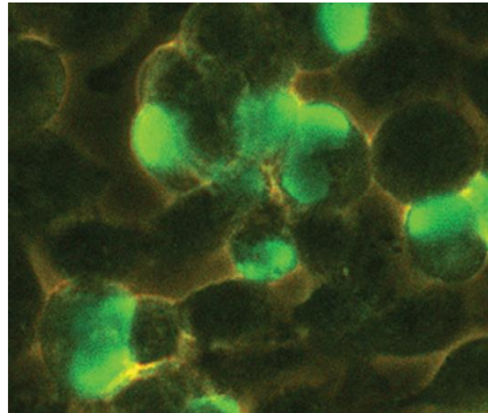

Figure 11. Indirect immunofluorescence to detect antibodies against *C. felis* [36].

1.3.8. Prevention and treatment

Vaccines for chlamydiosis are currently available for cats but not for other species [35]. Both inactivated and attenuated vaccines are available but only included as components of multivalent vaccine formulations. These vaccines typically also target feline herpesvirus, feline calicivirus, and feline parvovirus [36]. While feline chlamydial vaccines do not provide complete immunity, they may reduce the severity of disease and infection rates [35]. These are most useful in breeding catteries or animal shelters where it can be challenging to eliminate the organism [69]. Vaccination in kittens typically begins at 8 to 9 weeks of age, followed by a second dose administered 3 to 4 weeks later. For cats with ongoing risk of exposure, annual booster vaccinations are recommended [36].

Treatment is indicated to decrease clinical signs of infection and to eliminate carrier status and subsequent shedding of EBs [70]. Chlamydiosis in cats can be effectively managed with antibiotics, and improvement is typically observable within a few days. Studies or guidelines favour doxycycline therapy. Among tetracyclines, doxycycline has the advantage of requiring only a single daily dose and is most frequently used at a daily dosage of 10 mg/kg orally; however, in cases where a single daily dose results in vomiting, an alternative dosing strategy of 5 mg/kg administered orally twice daily may be employed. It is also reported that four weeks

should be sufficient for the complete elimination of the organism. Continuation of treatment is recommended for two weeks after the resolution of symptoms [36,70]. Although doxycycline proves to be the most effective option, it can pose potential side effects in young cats. If this is a concern, alternative antibiotics, such as enrofloxacin and pradofloxacin may be considered [71,72]. According to studies, azithromycin does not prove to be effective compared to doxycycline [63,73]. The prognosis is usually considered good with appropriate treatment [36].

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. All of the patients included in the study had underlying diseases. For the survey, patients were selected based on specific criteria, such as elevated levels of liver enzymes (ALT and AST), increased serum bilirubin, icterus, and the diagnosis of hepatitis of unknown origin. The study did not include patients with drug-induced liver injury, alcohol-related liver diseases, hepatitis due to genetic and metabolic disorders, and autoimmune hepatitis. Patients with increased liver enzymes due to other viral infections, including hepatitis A, B, and C and herpesviruses, were also excluded. 81 out of 1,383 patients had malignancy in their past medical history, 72 were immunocompromised on admission, 31 had chronic alcoholism, and 14 suffered from chronic renal failure. 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 [74]. 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 [74]. 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 $\chi2$ 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′) [75]; 0.2 µl 25 pmol Ch primer R (5′-GGAGTTAGCCGGTGCTTCTTTAC-3′) [75]; 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 real-time positive 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 real-time PCR, according to Helps *et al.* (2001) [64].

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) (Table 1).

Parameters	Variables	n	Anti-HEV IgG positive		Anti-HEV IgM positive	
			n	**(%)**	**n**	**(%)**
Sex	Males	664	208	31.3	47	7.1
	Females	719	221	30.7	23	3.2
Age group	1-10	36	3	8.3	0	0.0
	11-20	64	9	14.1	0	0.0
	21-30	126	19	15.1	1	0.8
	31-40	164	24	14.6	2	1.2
	41-50	214	66	30.8	6	2.8
	51-60	229	91	39.7	15	6.5
	61-70	273	111	40.6	15	5.5
	71-80	191	81	42.4	20	10.5
	81<	86	25	29.1	11	12.8
	Years	n	Seroprevalence		Acute infection	
			n	**(%)**	**n**	**(%)**
Number of tested patients	2018 [a]	256	96	37.5	10	3.9
	2019 [a]	645	177	27.4	35	5.4
	2020	482	156	32.4	25	5.2
	Total	**1,383**	**429**	**31**	**70**	**5.1**

Table 1. Analysis of epidemiological factors associated with IgG and IgM antibodies to hepatitis E virus, Szeged, Hungary, 2018/20. [a] Significant difference in anti-HEV IgG seropositivity (P = 0.003)

The age and gender distribution of tested patients were similar in 2018 and 2019 (Table 2). There was no significant difference in seropositivity between sexes in the affected population (P = 0.8163, RR = 1.019, CI = 0.8708–1.192) (Table 1). Most sera with anti-HEV IgG positivity were collected from adults and elderly patients with a mean age of 60 (range 1–98) years (Fig. 12). 87.2% of seropositive patients (n = 374) were above the age of 40 years (Table 1). Our results indicated that the most affected cohort in the seropositive population was the 71–80 age group (Fig. 12). The anti-HEV IgG seroprevalences in the 71–80 age group were significantly higher than those under 50 and over 80 (Supplementary Table 1). Comprehensive collation of seroprevalence results (P value, RR, and its CI) are shown in Supplementary Table 1.

Age group (yr)	Male (%)		Female (%)	
	2018	2019	2018	2019
0-10	4 (3.45)	2 (0.64)	5 (3.57)	9 (2.71)
11-20	2 (1.72)	13 (4.15)	8 (5.71)	20 (6.02)
21-30	12 (10.34)	36 (11.50)	4 (2.86)	36 (10.84)
31-40	17 (14.66)	39 (12.64)	23 (16.43)	35 (10.54)
41-50	20 (17.24)	60 (19.17)	14 (10.00)	44 (13.25)
51-60	18 (15.52)	47 (15.02)	25 (17.86)	60 (18.07)
61-70	26 (22.41)	49 (15.65)	30 (21.43)	69 (20.78)
71-80	15 (12.93)	42 (13.42)	22 (15.71)	42 (12.65)
>81	2 (1.72)	25 (7.99)	9 (6.43)	17 (5.12)

Table 2. The age and gender distribution of tested patients in 2018 and 2019, Szeged, Hungary.

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) (Table 1). Acute infections mainly occurred among middle-aged and elderly patients with a mean age of 63 (Fig. 13). Cases were confirmed primarily over the age of 40 (n = 67; 95.7%) (Table 1). 81 < age group was identified as the highest risk group in the anti-HEV IgM-positive population (Fig. 13). 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

(Supplementary Table 2). Differences between age groups are fully presented in Supplementary Table 2.

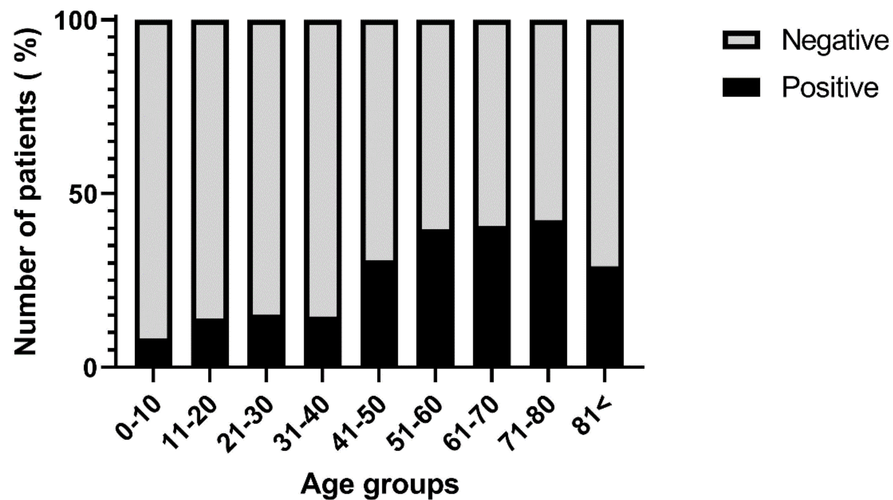

Figure 12. Comparison of anti-HEV IgG seroprevalences sorted by age, Szeged, Hungary, 2018/20. The vertical axis shows the number of patients (%) with (black bar) positive anti-HEV IgG or without (white bar) anti-HEV IgG. The horizontal axis shows patient age groups.

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 (Fig. 14). The differences were significant in January and July compared to August-December (Supplementary Table 3). There were no significant differences (P = 0.6359) between years in case of acute infections during the sampling period (Table 1). All significant differences between the sampling months can be found in Supplementary Table 3.

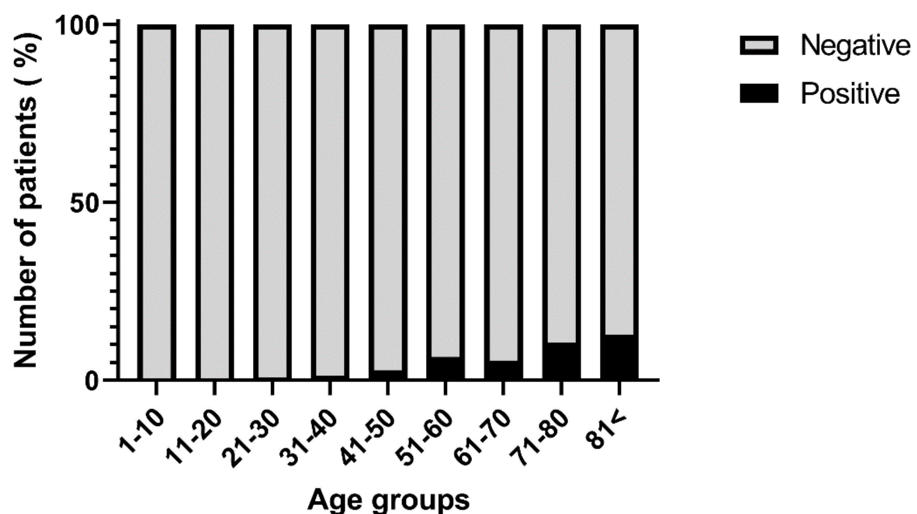

Figure 13. Comparison of acute HEV infections sorted by age, Szeged, Hungary, 2018/20. The vertical axis shows the number of patients (%) with (black bar) positive anti-HEV IgM or without (white bar) anti-HEV IgM. The horizontal axis shows patient age groups.

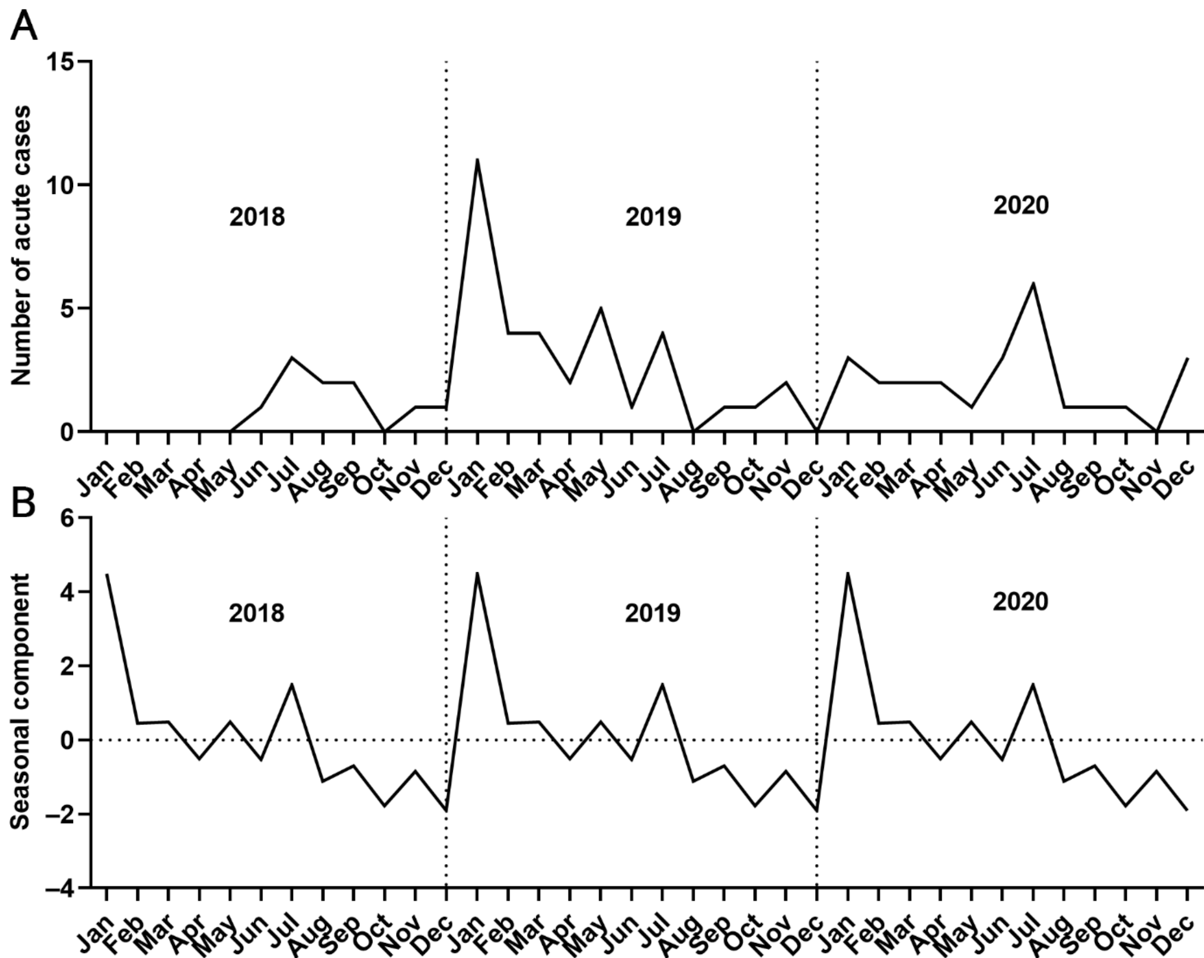

Figure 14. Time series analysis. The number of acute cases across three years (A) and the underlying seasonal component (B), Szeged, Hungary, 2018/20. The seasonal component is a part of the variations in a time series representing intra-year fluctuations that are more or less stable year after year concerning timing, direction, and magnitude. A positive value indicates a positive deviation from the trend line at any time and vice versa.

Out of 70 patients with acute HEV infections, 33 detailed medical histories were available. Three patients required albumin dialysis due to severe manifestations of HEV infection. One patient had hematologic malignancy, one patient with chronic renal failure received hemodialysis, and one patient had chronic alcoholism. Twenty-nine patients received only supportive treatment; in the case of 38 patients, no specific therapies were found in their medical records. Sixty-seven patients recovered, and three patients who received albumin dialysis have also recovered after long-term hospitalisation. No patient died because of hepatitis E viral infection or its consequences. Four out of 33 patients with detailed medical history had no underlying disease. While others had one or multiple underlying disorders; namely, 19 patients had hypertension, 12 had diabetes mellitus, 9 had a cardiologic disorder, 6 had malignancy, 4 had kidney disease, and 2 had cirrhosis. Eight patients were severely immunocompromised. In 17 patients, we could not identify the consumption of pork products or meat and alcohol. Five patients ate pork, sheep, deer, and seafood before developing symptoms and signs. Six patients

with HEV had chronic alcoholism, and five reported that they drank alcohol occasionally. Serum ALT ranged between 30 and 13,330 U/L, while serum bilirubin levels were 40-605 µmol/L.

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. First, HEV PCR from plasma and faecal samples from a patient with myeloproliferative disorder and thrombosis gave positive results, but after 13 days, both were negative. The second hemodialysis patient with severe HEV infection had only faecal HEV PCR positivity, and plasma was negative for HEV RNA. The previously mentioned method determined sequences after cDNA was detected in seven cases. 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. The comprehensive results of the sample collection are shown in Table 3. 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.

Sources	Symptomatic animals	Asymptomatic animals	Total
Cat shelter (cats)	4 (4.3%)/8; Ct: 34.0	12 (12.9%)/35; Ct: 37.5	16 (17.2%)/43
Veterinary clinic (cats)	8 (8.6%)/20; Ct: 36.2	0 (0.0%)/0	8 (8.6%)/20
Veterinary clinic (dogs)	6 (6.5%)/13; Ct: 35.1	0 (0.0%)/0	6 (6.4%)/13
Household pets (cats)	1 (1.1%)/13; Ct: 38.1	1 (1.1%)/2; Ct: 38.8	2 (2.2%)/15
Household pets (dogs)	0 (0.0%)/2	0 (0.0%)/0	0 (0.0%)/2
Total[1]	**19 (20.4%)**	**13 (14.0%)**	**32 (34.4%)**
Total[2]			**93 (100.0%)**

Table 3. Data of animals with positive pan-chlamydia PCR results with average Ct values, including the number of animals and their percentage distribution, sorted by relevant parameters. Symptomatic and asymptomatic columns show the ratio of PCR-positive/examined animals with the average Ct values.
[1] Sum of the values in the cells corresponding to columns. [2] All individuals participating in the study and the basis of comparison in this table.

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. Detailed data of individuals with positive pan-chlamydia PCR results are presented in Table 3. 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. This is attributed to the fact that animals at the clinic arrived with symptoms to initiate veterinary care. In the household pets category, samples from owners were usually submitted for testing when symptoms were observed in the animals. Often, these samples were from untreated animals that had not received veterinary care. Ct and Tm values of positive pan-chlamydia PCR results are detailed in Supplementary Table 4**.** The *C. felis*-specific PCR gave positive confirmatory results in 13 cases. 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* (Supplementary Fig. 1); 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 (Supplementary Fig. 2). 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. A comprehensive summary of identified bacteria and fungi is provided in Supplementary Table 5. 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. Twenty-seven samples (10 symptomatic cats, 11 asymptomatic cats, and six symptomatic dogs) proved negative by culture; in 10 cases, pan-chlamydia PCR gave positive results.

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

The understanding of HEV infection patterns among patients in Hungary is not well-documented. 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) to 31.0% (2018-2020 period) [22]. 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 [22]. The observed rise in IgG seropositivity may partly be attributed to the increased use of a highly sensitive ELISA assay (WANTAI Bio-Pharm, China) [76]. When comparing available data, seroprevalence rates in East-Central European countries appear to be fairly consistent. For instance, the reported HEV seroprevalence among the general population in neighbouring countries is 17.8% in Slovakia (DRG Instruments), 15% in Serbia (in-house assay), 14% in Romania (Mikrogen), and 14.3% in Austria (Wantai) [77-79]. However, interpreting these findings and comparing results is challenging due to the use of different diagnostic kits and diverse patient populations. Previous studies have identified the consumption of raw or undercooked pork as the primary route of HEV transmission in developed countries [80,81]. In our study, only five patients provided detailed information about their eating habits, including pork, sheep, deer, and seafood consumption, as potential sources of HEV infection. Other patients had also consumed meat before the onset of acute HEV, but specific details regarding the meat type and timing were unavailable. 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 [82,83].

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 [15,17,84]. Hungary has consistently been identified as a country with severe liver-related issues associated with heavy alcohol use [85]. Previous research highlights that alcohol-induced liver damage is

common among middle-aged and older men [86]. 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%) [22]. In addition, anti-HEV IgG-positive cases also occurred in individuals over 40 years old (n = 374, 87.2%). 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 [15,17,84].

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 [9,22,87,]. 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 [22]. The proportion of acute HEV infections detected through serological testing in our study (5.1%) was lower compared to the prior survey (9.6%) [22]. This deviation may be partially attributed to the lower specificity of the anti-HEV IgM ELISA assay of the earlier study [22].

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% [22]. 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 [10].

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 [6]. In Hungary, prior research predominantly identified subgenotype 3e strains [22].

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 [47,88]. 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. Although symptomatic animals can also spread the infection, they are more likely to receive treatment. 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 [47,88-90]. Increased awareness could lead to more appropriate treatments, reducing the disease's incidence and the risk of further transmission.

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 [30]. 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%) [30]. 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%) [30]. Other Central European studies have also reported high positivity rates in stray and shelter cat populations. In a study in Romania, the positivity rate was 65.3%, while in a study in Slovakia, the positivity rates were 35.7% and 31.0%, respectively [54,91]. These results indicate that the positivity rates vary across different animal populations and environmental settings. Nevertheless, shelter and stray cat populations and subgroups appear to have the highest PCR positivity rates.

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 [92-94]. Our findings align with previous findings, identifying *Pseudomonas*, *Staphylococcus, Enterococcus, Klebsiella, Pantoea,* and *Bacillus* as important pathogens of conjunctivitis among pets [92-95]. A significant colonisation rate (27 out of 101 samples, 26.7%) was observed, similar to earlier studies [92]. 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 [54]. 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 [96]. 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 [96].

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 [36,70]. 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.

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10. SUPPLEMENTARY MATERIALS

Supplementary Table 1. Comparison of the risk for anti-HEV IgG positivity in the age groups.

Age groups	0-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81<
0-10		P = 0.5288. RR=1.688 (CI=0.5379 to 5.559)	P=0.4119. RR=1.810 (CI=0.6305 to 5.588)	P=0.4245. RR=1.756 (CI=0.6228 to 5.356)	**P=0.0043. RR=3.701 (CI=1.381 to 10.89)**	**P=0.0001. RR=4.769 (CI=1.794 to 13.96)**	**P<0.0001. RR=4.879 (CI=1.841 to 14.269**	**P<0.0001. RR=5.089 (CI=1.912 to 14.91)**	**P=0.0170. RR=3.488 (CI=1.252 to 10.53)**
11-20			P>0.9999. RR=1.072 (CI=0.5302 to 2.228)	P>0.9999. RR=1.041 (CI=0.5258 to 2.117	**P=0.0097. RR=2.193 (1.206 to 4.188)**	**P<0.0001. RR=2.826 (CI=1.575 to 5.341)**	**P<0.0001. RR=2.892 (CI=1.619 to 5.446)**	**P<0.0001. RR=3.016 (CI=1.678 to 5.7079**	**P=0.0320. RR=2.067 (CI=1.069 to 4.126)**
21-30				P>0.9999. RR=0.9705 (CI=0.5622 to 1.685)	**P<0.0012. RR=2.045 (CI=1.310 to 3.258)**	**P<0.0001. RR=2.635. (CI=1.719 to 4.136)**	**P<0.0001. RR=2.692 (CI=1.770 to 4.211)**	**P<0.0001. RR=2.812 (Ci=1.830 to 4.421)**	**P=0.0161. RR=1.928 (CI=1.141 to 3.260)**
31-40					**P=0.0002. RR=2.107 (CI=1.398 to 3.220)**	**P<0.0001. RR=2.715 (CI=1.873 to 4.081)**	**P<0.0001. RR=2.778 (CI=1.892 to 4.153)**	**P<0.0001. RR=2.898 (CI=1.955 to 4.364)**	**P=0.0076. RR=1.986 (CI=1.211 to 3.238)**
41-50						P=0.0589. RR=1.288 (CI=0.9995 to 1.6689	**P=0.0290. RR=1.318 (CI=1.034 to 1.692)**	**P=0.0174. RR=1.375 (CI=1.062 to 1.785)**	P=0.7833. RR=0.9426 (CI=0.6337 to 1.366)
51-60							P=0.8553. RR=1.023 8CI=0.8268 to 1.270)	P=0.6187. RR=1.067 (CI=0.8471 to 1.342)	P=0.0891. RR=0.7215 (CI=0.5004 to 1.036)
61-70								P=0.7739. RR=1.043 (CI=0.8357 to 1.295)	P=0.0568. RR=0.7150 (CI=0.4919 to 1.005)
71-80									**P=0.0447. RR=0.6855 (CI=0.4680 to 0.9744)**

Abbreviations: P, *P* value; RR, relative risk; CI, confidence interval for RR; The significant differences are indicated in bold.

Supplementary Table 2. Comparison of the risk for anti-HEV IgM positivity in the age groups.

Age groups	1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81<
1-10		P > 0.9999	P > 0.9999	P > 0.9999	P = 0.5976	P = 0.2350	P= 0.2318	**P = 0.0498**	**P = 0.0325**
11-20			P > 0.9999	P > 0.9999	P = 0.3419	**P = 0.0477**	P = 0.0844	**P = 0.0052**	**P = 0.0026**
21-30				P>0.9999. RR = 1.537 (CI=0.2035 to 11.67)	P = 0.2661. RR= 3.533 (CI=0.5696 to 22.25)	**P=0.0136. RR=8.253 (CI=1.429 to 48.74)**	**P=0.0269. RR=6.923 (CI=1.198 to 40.90)**	**P=0.0003. RR=13.19 (CI=2.315 to 77.00)**	**P=0.0003. RR=16.12 (CI=2.751 to 96.22)**
31-40					P = 0.743. RR = 2.299 (CI=0.5395 to 9.885)	**P=0.0108. RR=5.371 (CI=1.400 to 20.92)**	**P=0.0376. RR=4.505 (CI=1.174 to 17.56)**	**P=0.0002. RR=8.586 (Ci=2.285 to 32.83)**	**P=0.0002. RR=10.49. (CI=2.676 to 41.55)**
41-50						P=0.0749. RR=2.366 (CI=0.9558 to 5.750)	P=0.1800. RR=1.960 (CI=0.8010 to 4.831)	**P=0.0020. RR=3.735 (CI= 1.581 to 8.892)**	**P=0.0016. RR=4.562 (CI=1.799 to 11.54)**
51-60							P=0.7064. RR=0.8388 (CI=0.4249 to 1.658)	P=0.1596. RR=1.599 (0.8511 to 3.066)	P=0.1049. RR=1.953 (0.9414 to 3.989)
61-70								P=0.0507. RR=1.906 (CI=1.012 to 3.589)	**P=0.0311. RR=2.328 (CI=1.120 to 4.761)**
71-80									P=0.5451. RR=1.222 (CI=0.6149 to 2.382)

Abbreviations: P, *P* value; RR, relative risk; CI, confidence interval for RR; The significant differences are indicated in bold.

Supplementary Table 3. Significant differences in occurrence between months in the sampling period*,**.

Months	January	March	May	June	July
Signifi-cance	August (0.006) December (0.027) November (0.009) October (0.0001) September (0.005)	October (0.018)	October (0.031)	October (0.036)	August (0.012) December (0.047) November (0.017) October (0.001) September (0.011)

* Only significant differences are shown in this table. *P* values are shown in brackets.
** For each significant pair, the name of the month with the smaller proportion appears in the column of the month with a larger proportion.

Supplementary Table 4. Table of positive pan-chlamydia PCR results with relevant details.

Table of positive pan-chlamydia PCR results with relevant details.

ID number	Ct values	Tm values	Evaluation
50586	40.00	84.2	positive
50907	34.80	84.8	positive
50908	37.60	81.3	positive
50909	28.50	81.6	positive*
50911	38.10	84.8	positive
51526	39.10	84.0	positive
51528	37.80	83.9	positive
51529	39.50	81.6	positive
53342	38.90	80. 0	positive
53340	39.70	82.9	positive
53339	38.30	81.6	positive
56423	35.40	85.1	positive
56411	38.90	83.0	positive
56410	39.20	85.2	positive
56413	35.50	84.6	positive
56918	39.90	84.8	positive
57394	37.50	82.5	positive
57895	38.80	83.1	positive
58538	21.20	82.1	positive*
151096	34.10	81. 9	positive
151102	35.00	84. 8	positive
151105	38.40	83. 7	positive
151108	38.20	82.0	positive
151336	40.00	81.0	positive
151338	39.50	81.4	positive
151339	39.50	81.3	positive
151671	38.50	82.8	positive
151673	37.70	82.0	positive
152373	28.90	81.7	positive*
152370	24.50	81.7	positive*
152868	35.60	81.3	positive
152875	35.20	82.2	positive
152876	34.60	81.8	positive

**C. felis* was detected by sequence analysis and confirmation by *C. felis* real-time PCR.

Supplementary Table 5. Table of identified bacteria and funghi as a result of the culture-based examinations.

The distribution of identified species categorised by genera.

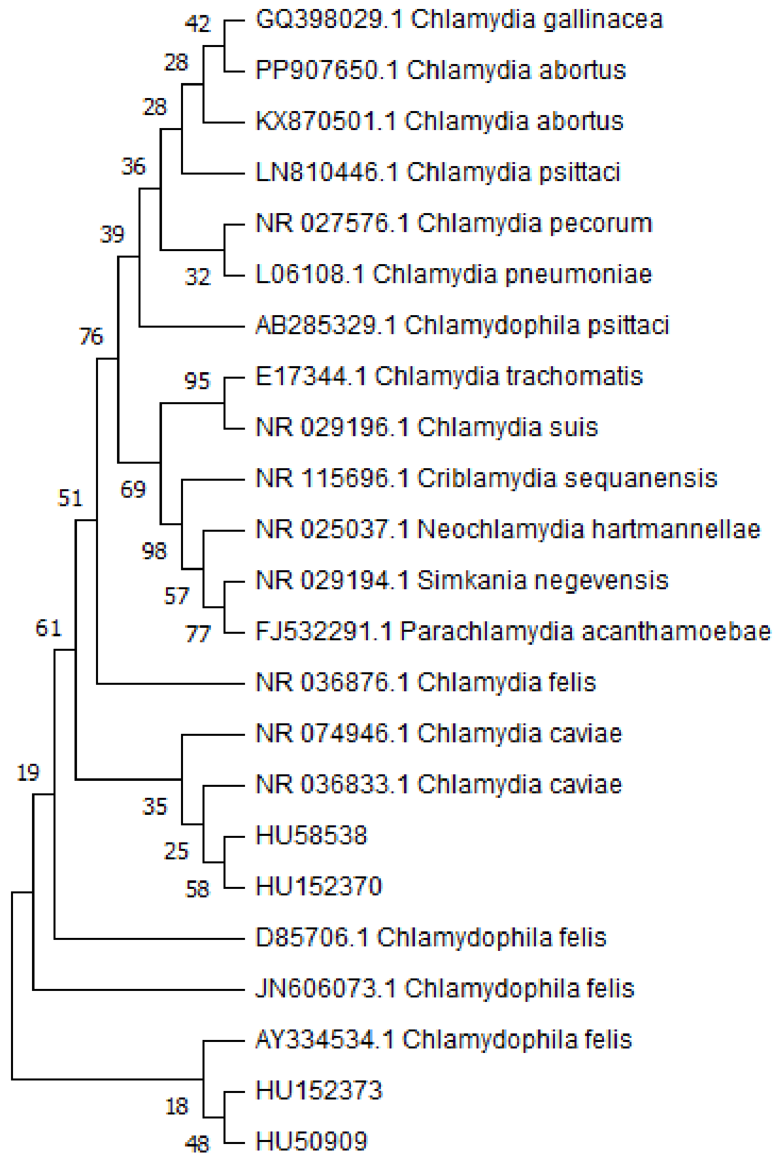
Bacteria			
Genus	**Species**	**Total (n)***	**%**
Achromobacter	*spanius* (1) **1-,1C,1H**	1	0.7
Acinetobacter	*pittii (1)* **1+,1C,1S**; *johnsonii (4)* **4-,4C,4S**; *schindleri (1)* **1+,1D,1V**; *radioresitens (1)* **1+,1C,1H**; *calcoaceticus (1)* **1-,1C,1H**; *ursingii (2)* **2+,1C,1D,2V**; *lwoffii (3)* **3-,3C,2S,1H**; *beijerinckii (1)* **1+,1D,1V**; *lactucae (1)* **1+,1C,1V**	15	9.8
Aerococcus	*viridans (2)* **2-,2C,2S**	2	1.3
Aeromonas	*caviae (2)* **1+,1-,2C,2S**; *veronii (1)* **1-,1C,1S**; *hydrophila (1)* **1-,1C,1S**	4	2.6
Agromyces	*bracchium (1)* **1-,1C,1H**	1	0.7
Bacillus	*cereus (2)* **2+,2C,1H,1S**; *pumilus (2)* **2+,2C,1V,1H**; *mycoides (1)* **1+,1D,1V**; *sonorensis (1)* **1+,1C,1H**	6	3.9
Brachybacterium	*conglomeratum (1)* **1-,1C,1S**	1	0.7
Brevundimonas	*diminuta (3)* **3+,1C,2D,3V**; *vesicularis (1)* **1+,1C,1V**; *sp. (1)* **1-,1C,1H**	5	3.3
Cellulomonas	*uda (1)* **1+,1D,1V**; *pakistanensis (1)* **1+,1D,1V**	2	1.3
Clostridium	*colicanis (1)* **1+,1C,1S**; *perfringens (1)* **1-,1C,1S**	2	1.3
Comamonas	*testosteroni (1)* **1+,1C,1S**	1	0.7
Corynebacterium	*mastitidis (2)* **1+,1-,2C,1S,1V**; *spheniscorum (1)* **1-,1C,1S**	3	2.0
Cutibacterium	*acnes (1)* **1-,1C,1S**	1	0.7
Enterobacter	*cloaceae (4)* **2+,2-,4C,3S,1H**	4	2.6
Enterococcus	*faecium (3)* **3+,3C,2S,1H**; *faecalis (4)* **3+,1-,4C,2S,2V**; *hirae (1)* **1+,1D,1V**; *italicus (1)* **1+,1C,1V**	9	5.9
Exiguobacterium	*mexicanum (1)* **1+,1D,1V**; *sp. (2)* **2+,2C,1V,1H**	3	2.0
Glutamicibacter	*arilaitensis (3)* **1+,2-,3C,2S,1V**; *protophormiae (1)* **1-,1C,1S**	4	2.6
Klebsiella	*pneumoniae (3)* **3+,2C,1D,2S,1V**; *aerogenes (1)* **1+,1D,1V**	4	2.6
Kurthia	*gibsonii (1)* **1-,1C,1S**	1	0.7
Lactococcus	*lactis (1)* **1+,1D,1V**; *raffinolactis (1)* **1-,1C,1S**	2	1.3
Lelliottia	*amnigena (1)* **1+,1C,1H**	1	0.7
Leuconostoc	*mesenteroides (1)* **1+,1C,1V**	1	0.7
Lysinibacillus	*fusiformis (1)* **1-,1C,1S**; *xylanilyticus (1)* **1+,1C,1V**	2	1.3
Massilia	*sp. (1)* **1-,1C,1S**	1	0.7
Microbacterium	*testaceum (1)* **1+,1D,1V**; *neimengense (1)* **1+,1C,1H**; *phyllosphaerae (1)* **1-,1C,1S**; *esteraromaticum (1)* **1-,1C,1S**; *oxydans (1)* **1+,1D,1V**; *paraoxydans (2)* **2+,2C,2V**; *foliorum (1)* **1+,1D,1V**; *liquefaciens (1)* **1+,1C,1V**; *luteus (1)* **1+,1C,1V**	10	6.5
Moraxella	*canis (1)* **1+,1D,1V**	1	0.7
Morganella	*morganii (1)* **1+,1D,1V**	1	0.7
Paenarthrobacter	*ilicis (1)* **1-,1C,1S**	1	0.7
Paenibacillus	*amylolyticus (2)* **1+,1-,1C,1D,1S,1V**	2	1.3
Pantoea	*agglomerans (5)* **5+,1C,4D,5V**	5	3.3

Proteus	*sp. (1)* **1+,1D,1V**	1	0.7
Pseudarthrobacter	*chlorophenolicus (2)* **2+,2C,2H**; *scleromae (1)* **1+,1C,1V**; *oxydans (1)* **1-,1C,1S**	4	2.6
Pseudescherichia	*vulneris (1)* **1+,1D,1V**	1	0.7
Pseudomonas	*aeruginosa (2)* **2+,2C,2V**; *koreensis (5)* **5+,3C,2D,4V,1H**; *corrugata (1)* **1+,1C,1H**; *taetolerans (1)* **1+,1C,1H**; *azotoformans (1)* **1+,1C,1H**; *extremorientalis (1)* **1+,1C,1H**; *agarici (1)* **1-,1C,1S**; *fulva (2)* **2+,1C,1D,1V,1H**; *rhodesiae (1)* **1+,1C,1H**; *chlororaphis (1)* **1+,1C,1H**; *flavescens (1)* **1-,1C,1S**	17	11.1
Psychrobacter	*pulmonis (2)* **2-,2C,2S**; *sanguinis (1)* **1+,1D,1V**	3	2.0
Rothia	*nasimurium (1)* **1+,1C,1V**	1	0.7
Serratia	*rubidaea (1)* **1-,1C,1S**	1	0.7
Sporosarcina	*ureae (1)* **1-,1C,1S**	1	0.7
Staphylococcus	*aureus (1)* **1+,1D,1V**; *felis (7)* **5+,2-,7C,3S,2V,2H,**; *epidermidis (3)* **2+,1-,3C,2S,1V**; *simulans (1)* **1+,1C,1V**; *sciuri (1)* **1+,1D,1V**; *haemolyticus (2)* **2-,2C,2S**; *pseudintermedius (1)* **1+,1D,1V**	16	10.5
Stenotrophomonas	*nitritireducens (1)* **1+,1C,1H**; *rhizophila (1)* **1+,1D,1V**	2	1.3
Streptococcus	*peroris (1)* **1+,1C,1V**; *sanguinis (1)* **1+,1C,1V**	2	1.3
Streptomyces	*naganishii (1)* **1+,1C,1V**	1	0.7
	Funghi		
Aspergillus	*flavus (1)* **1-,1C,1S**	1	0.7
Candida	*parapsilosis (1)* **1+,1C,1H**	1	0.7
Malassezia	*pachydermatis (2)* **2+,1C,1D,1S,1V**	2	1.3
Sarocladium	*strictum (1)* **1+,1D,1V**	1	0.7
Syncephalastrum	*racemosum (1)* **1+,1D,1V**	1	0.7
Trichophyton	*rubrum (1)* **1+,1C,1V**	1	0.7
Total		153	100.0

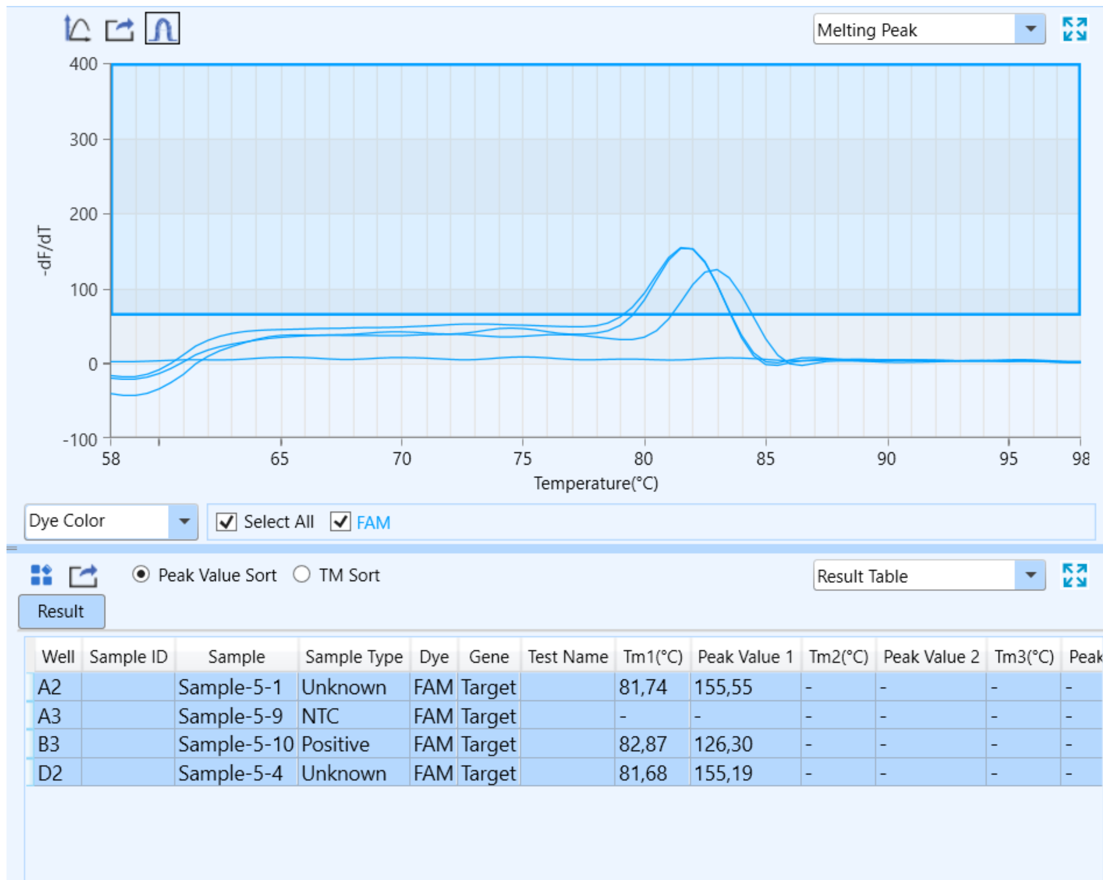
*number of species.

+, -, C, D, S, V, H indicate the main categories compared in the study. Microorganisms were characterised by these markers. +: symptomatic animal; **-**: asymptomatic animal; **C**: cats; **D**: dogs; **S**: cat shelter; **V**: veterinary clinic; **H**: household pets

Supplementary Figure 1. Phylogenetic tree based on the sequence analysis of pan-chlamydial PCR product. The phylogenetic tree was constructed with MEGA11 using the maximum likelihood method (bootstrap values 1,000). HU58538, HU152370, HU152373, and HU50909 are indicated as Hungarian data.



Supplementary Figure 2. Melting analysis of pan-chlamydial PCR products using Gentier96E real-time PCR instrument (Xian Tianlong Science and Technology Co., Ltd, China). Samples 5-1 and 5-4 were *Chlamydia felis* positive samples confirmed by *C. felis*-specific PCR; positive control was *C. trachomatis* with Tm value 82.8.



Well	Sample ID	Sample	Sample Type	Dye	Gene	Test Name	Tm1(°C)	Peak Value 1	Tm2(°C)	Peak Value 2	Tm3(°C)	Peak
A2		Sample-5-1	Unknown	FAM	Target		81,74	155,55	-	-	-	-
A3		Sample-5-9	NTC	FAM	Target		-	-	-	-	-	-
B3		Sample-5-10	Positive	FAM	Target		82,87	126,30	-	-	-	-
D2		Sample-5-4	Unknown	FAM	Target		81,68	155,19	-	-	-	-

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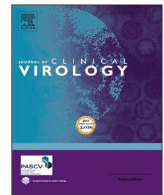

Characteristics of hepatitis E viral infections in Hungary

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ABSTRACT

Background: Hepatitis E virus (HEV) is one of the most important causes of hepatitis worldwide. Despite this, limited data published more than ten years ago are only available about HEV epidemiology in Hungary.
Objectives: We aimed to determine the epidemiological features of HEV infections among patients submitted to various departments of our university hospital in Hungary with signs and symptoms referring to acute hepatitis.
Study design: One thousand four hundred thirty-one sera samples from 1,383 patients were analyzed by enzyme-linked immunosorbent assays (ELISA). In some patients, HEV RNA was detected by broad-range nested polymerase chain reaction (PCR) if acute hepatitis was confirmed. PCR products were sequenced and compared with other available sequences in GenBank.
Results: Five hundred eighteen sera from 429 patients proved positive (31.0%) for HEV-specific immunoglobulin G (IgG) with a mean age of 60.0 years. Most sera with anti-HEV IgG antibodies were collected from adults and elderly patients. Anti-HEV IgM positive results were found in the case of 95 sera samples from 70 patients (5.1%). Acute HEV infections were confirmed mostly over 40 ($n = 67$, 95.7%). The number of males ($n = 47$, 67.1%) was higher than females ($n = 23$, 32.9%). We detected HEV-specific PCR products in seven patients (10.9%). Genotyping was successful for 5 out of 7 PCR-positive samples. All sequences belonged to genotype 3 (subgenotypes: e, f).
Conclusions: In our survey, we confirmed the constant presence of acute HEV infections in Hungary and an increased seroprevalence of anti-HEV IgG antibodies compared to a previous study.

1. Background

Hepatitis E virus (HEV) is a non-enveloped, single-stranded RNA virus. HEV belongs to the *Orthohepevirus* genus within the family *Hepeviridae*. Hepatitis E virus is one of the most important causes of acute hepatitis worldwide. It is estimated that 20 million HEV infections and 70 000 deaths occur yearly [1]. The virus causes acute and self-limiting infections with a low mortality rate. However, fulminant hepatitis can occasionally develop in some patients, mostly older men, which is usually associated with underlying chronic liver disease [2]. Pregnant women also belong to a high-risk group. In this case, the mortality can reach 30%, while this rate is only between 0.2 and 4% in a general population [3]. HEV infections may also have various extrahepatic manifestations, such as Guillain-Barré syndrome or encephalitis [4,5].

International studies confirmed that the incidence of hepatitis E infection has increased in many European countries over the last ten years [6–8]. In Europe, 5–15% of acute hepatitis of unknown origin is associated with HEV infection; however, the seroprevalence is varied from country to country. In addition to this, regional differences in some countries could be observed. For example, in France, seroprevalences range from 8 to 86.4%, and in Spain, values vary from 0.8 to 7.3% [3].

1.1. Objective

In Hungary, limited data are only available about HEV epidemiology. These results were published more than ten years ago, and only a few studies include comprehensive epidemiological surveys [9,10]. Because of these facts, it would be necessary to perform novel surveys that characterize Hungary's current epidemiological status. We aimed to determine the features of HEV infections among patients submitted to

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our University Hospitals with symptoms of acute hepatitis.

2. Study design

2.1. HEV serology

Between May 2018 and December 2020, 1431 sera samples from 1383 patients admitted to various departments (60% from Infectious Diseases and Gastroenterology and Hepatology Departments, and 40% from other outpatient departments of the University (Hematology, Cardiology, Nephrology, Dialysis Center etc., if the patient had underlying diseases)) of the University of Szeged, Albert Szent-Györgyi Health Center (Szeged, Hungary) were analyzed. Patients were selected for this survey based on increased ALT and/or AST, increased serum bilirubin, icterus, and the diagnosis of hepatitis of unknown origin. This study did not include patients with drug-induced liver injury, alcoholic liver diseases, hepatitis due to genetic and metabolic disorders, and autoimmune hepatitis. Patients with increased liver enzymes due to other viral infections, including hepatitis A, B, and C, and herpesviruses were also excluded. 81 out of 1383 patients had malignancy in their past medical history, 72 were immunocompromised on admission, 31 had chronic alcoholism, and 14 suffered from chronic renal failure. 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 case of a positive sample for anti-HEV IgM with EIA, VIDAS enzyme-linked fluorescence assay (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 analyzed for the presence of HEV RNA.

2.2. Detection and sequencing of hepatitis E virus RNA

According to the manufacturer's instructions, viral RNA from stool was extracted using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Germany). Viral RNA from blood was purified using QIAsymphony DSP Virus/Pathogen Kit (Qiagen, Germany) in combination with the QIAsymphony SP (Qiagen, Germany). HEV RNA was amplified by a broad-range nested PCR method using previously published primers [11]. The amplified cDNA products were detected on a 1.5% agarose gel using ECO Safe Nucleic Acid Staining Solution (Pacific Image Electronics) at 90 V for 45 min. Results were visualized and documented by the PXi Touch Multi-Application Gel Imaging System (Syngene, United Kingdom). 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). PCR products were sequenced using GenomeLab DTCS – Quick Start Kit (Beckman Coulter, US) according to the manufacturer's instructions with previously published primers [11]. Sequencing and analysis were performed on a GenomeLab GeXP Genetic Analysis System (Beckman Coulter, US). 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.

2.3. Statistical analyses

Analyses were performed 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, US). 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. For more than two categorical variables, column proportions were compared using a z-test. $P < 0.05$ 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 were conducted using the R 3.0.1 program language (Boston, MA, US). Graphs were created using GraphPad Prism 8.4.3 (San Diego, CA, US).

3. Results

3.1. Seroprevalence study

Since May 2018, 1431 serum samples from 1383 patients admitted to outpatient departments for various years were analysed. Thirty-three patients had multiple longitudinal samples. In the case of 9 out of 33 patients, physicians submitted multiple samples during the course of acute HEV infections; we detected both anti-HEV IgM and anti-HEV IgG in these specimens. In five patients, because of weak positive IgM and negative IgG results to confirm acute HEV, multiple samples were also sent to the lab. 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; acute HEV infection was therefore excluded in these symptomatic patients.

Regarding the sampling period, we only found a significant difference in anti-HEV IgG seropositivity between 2018 and 2019 ($P = 0.003$) (Table 1). The age and gender distribution of tested patients were similar in 2018 and 2019 (Table 2). The difference in seropositivity between sexes was not found to be significant in the affected population ($P = 0.8163$, RR = 1.019, CI = 0.8708–1.192) (Table 1). Most sera with anti-

Table 1
Analysis of epidemiological factors associated with presence of IgG and IgM antibodies to hepatitis E virus, Szeged, Hungary, 2018/20.

Parameters	Variables	n	Anti-HEV IgG-positive		Anti-HEV IgM-positive	
			n	**(%)**	**n**	**(%)**
Sex	Males	664	208	31.3	47	7.1
	Females	719	221	30.7	23	3.2
Age group	1–10	36	3	8.3	0	0.0
	11–20	64	9	14.1	0	0.0
	21–30	126	19	15.1	1	0.8
	31–40	164	24	14.6	2	1.2
	41–50	214	66	30.8	6	2.8
	51–60	229	91	39.7	15	6.5
	61–70	273	111	40.6	15	5.5
	71–80	191	81	42.4	20	10.5
	81<	86	25	29.1	11	12.8
Number of tested patients	Years	n	Seroprevalence		Acute infection	
			n	**(%)**	**n**	**(%)**
	2018 [a]	256	96	37.5	10	3.9
	2019 [a]	645	177	27.4	35	5.4
	2020	482	156	32.4	25	5.2
	Total	**1383**	**429**	**31**	**70**	**5.1**

[a] Significant difference in anti-HEV IgG seropositivity ($P = 0.003$).

Table 2
The age and gender distribution of tested patients in 2018 and 2019, Szeged, Hungary.

Age group (yr)	Male (%)		Female (%)	
	2018	2019	2018	2019
0–10	4 (3.45)	2 (0.64)	5 (3.57)	9 (2.71)
11–20	2 (1.72)	13 (4.15)	8 (5.71)	20 (6.02)
21–30	12 (10.34)	36 (11.50)	4 (2.86)	36 (10.84)
31–40	17 (14.66)	39 (12.64)	23 (16.43)	35 (10.54)
41–50	20 (17.24)	60 (19.17)	14 (10.00)	44 (13.25)
51–60	18 (15.52)	47 (15.02)	25 (17.86)	60 (18.07)
61–70	26 (22.41)	49 (15.65)	30 (21.43)	69 (20.78)
71–80	15 (12.93)	42 (13.42)	22 (15.71)	42 (12.65)
>81	2 (1.72)	25 (7.99)	9 (6.43)	17 (5.12)

HEV IgG positivity were collected from adults and elderly patients with a mean age of 60 (range 1–98) years (Fig. 1). 87.2% of seropositive patients ($n = 374$) were above the age of 40 years (Table 1). Our results indicated that the most affected cohort in the seropositive population was the 71–80 age group (Fig. 1). The anti-HEV IgG seroprevalences in the 71–80 age group were significantly higher than those under 50 and over 80 (Supplementary Table 1). Comprehensive collation of seroprevalence results (*P* value, RR, and its CI) are shown in Supplementary Table 1.

3.2. Evaluation of acute infection

Anti-HEV IgM positive results were found only in patients with common symptoms of acute hepatitis. Twelve (0.8%) out of 1383 patients proved to be false-positive for HEV-specific IgM. In the case of acute HEV infection (70 out of 1383 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) (Table 1). Acute infections mainly occurred among middle-aged and elderly patients with a mean age of 63 (Fig. 2). Cases were confirmed mostly over the age of 40 ($n = 67$; 95.7%) (Table 1). 81 < age group was identified as the highest risk group in the anti-HEV IgM positive population (Fig. 2). 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 (Supplementary Table 2). Differences between age groups are fully presented in Supplementary Table 2.

On average, six acute cases per month occurred in the sampling interval (May 2018–December 2020). Prevalence was higher in the first half of the year, where two significantly higher peaks were observed (Fig. 3). The differences were found to be significant in the case of January and July, compared to the August–December period (Supplementary Table 3). There were no significant differences (P = 0.6359) between years in case of acute infections during the sampling period

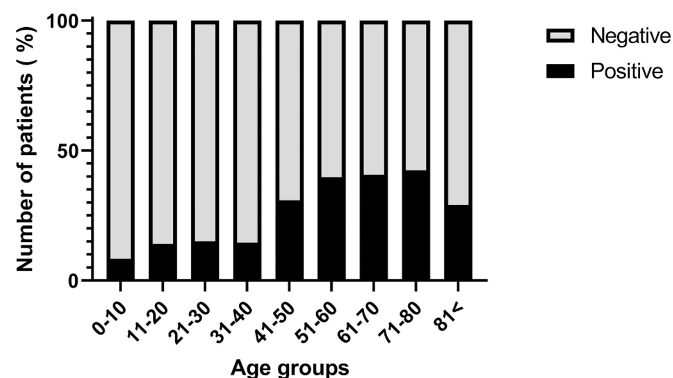

Fig. 1. The vertical axis shows the number of patients (%) with (black bar) positive anti-HEV IgG or without (white bar) anti-HEV IgG. The horizontal axis shows patient age groups.

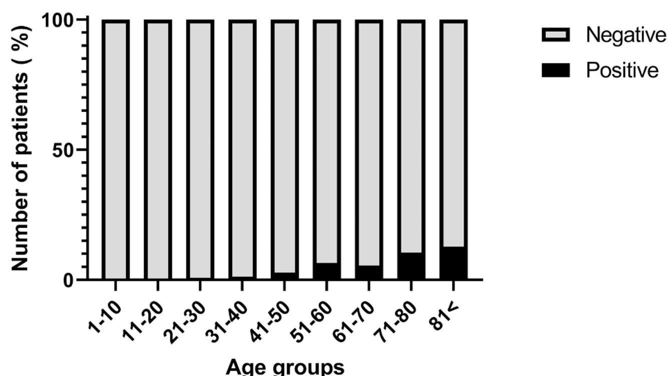

Fig. 2. The vertical axis shows the number of patients (%) with (black bar) positive anti-HEV IgM or without (white bar) anti-HEV IgM. The horizontal axis shows patient age groups.

(Table 1). All significant differences between the sampling months can be found in Supplementary Table 3.

In 33 patients with acute HEV infections out of 70, detailed past medical history was available for us. Three patients were treated with albumin dialysis due to severe manifestations of HEV infection. One patient had hematologic malignancy, 1 patient with chronic renal failure received hemodialysis and 1 patient suffered from chronic alcoholism. Twenty-nine patients received only supportive treatment, in the case of 38 patients no specific treatments were found in their medical records. Sixty-seven patients recovered, and 3 patients who received albumin dialysis have also recovered after long-term hospitalization. No patient died because of hepatitis E viral infection or its consequences.

Four out of 33 patients with detailed medical history had no underlying disease. While others had one or multiple underlying disorders; namely, 19 patients had hypertension, 12 had diabetes mellitus, 9 with a cardiologic disorder, 6 had malignancy, 4 had kidney disease, and 2 with cirrhosis. Eight patients were severely immunocompromised. In 17 patients, we could not identify consumption of pork products or meat and alcohol. Five patients ate pork, sheep, deer, and seafood before developing symptoms and signs. Six patients with HEV had chronic alcoholism, and five said they occasionally drink alcohol. Serum alanine aminotransferase ranged between 30 and 13,330 U/L, while serum bilirubin levels were between 40 and 605 μmol/L.

3.3. Detection and genotyping of HEV cDNA from stool

Total RNA was isolated from 75 stool and three plasma samples of 64 patients with IgM positive results. In the case of 7 patients, PCR gave positive results from feces; among these patients, only in one case we have detected viremia. 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 fecal 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' fecal specimens (10.9%) with a mean age of 70 years. All PCR-positive patients were above 50, and the eldest patient was 91. Six of 7 HEV PCR-positive patients had at least two consecutive fecal specimens; in all cases, ten days after the first fecal PCR positivity repeated PCR gave negative results. In the case of 2 patients who required albumin dialysis due to severe HEV infections, plasma specimens were also obtained. First HEV PCR from plasma and fecal samples from a patient with myeloproliferative disorder and thrombosis gave positive results, but after 13 days, both were negative. The second hemodialysis patient with severe HEV infection had only fecal HEV PCR positivity, and plasma was negative for HEV RNA. After cDNA was detected in seven cases, sequences were determined by the previously mentioned method. Genotyping was successful for 5 out of 7

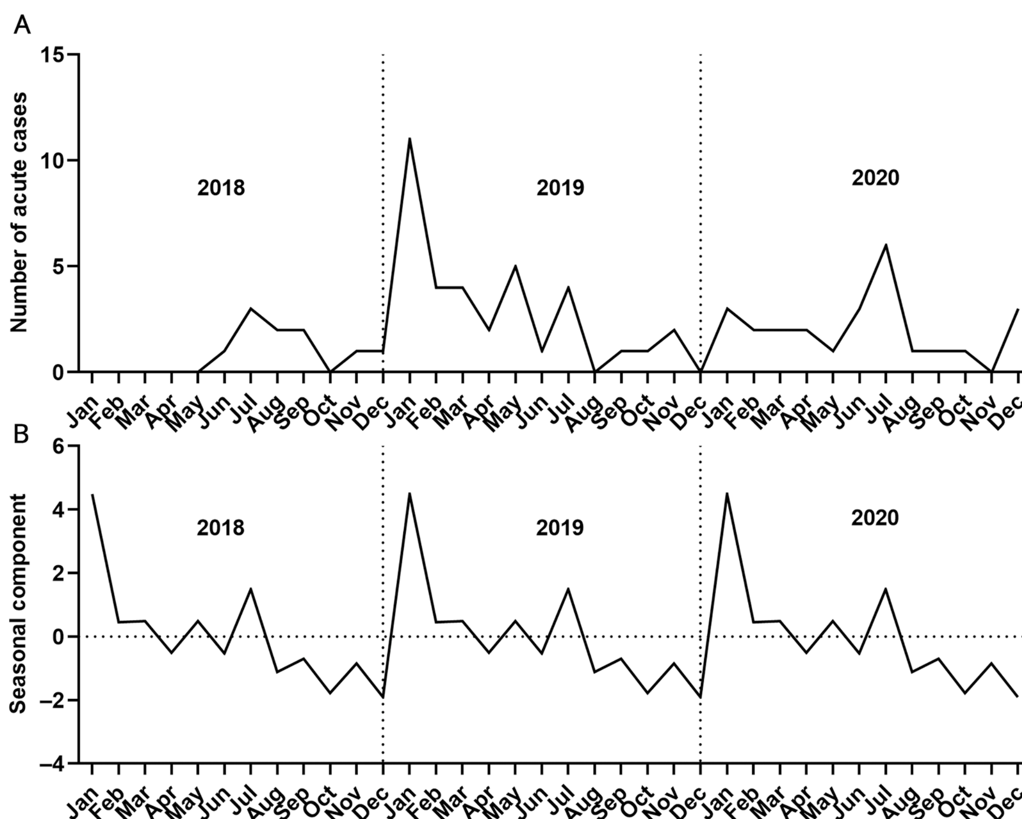

Fig. 3. Figure A shows the actual number of acute cases across by month, figure B shows the underlying seasonal component of prevalence fluctuations. The seasonal component is a part of the variations in a time series representing intra-year fluctuations that are more or less stable year after year with respect to timing, direction, and magnitude. A positive value indicates a positive deviation from the trend line at any time and vice versa.

PCR-positive samples. As a result of molecular characterization, five genotype 3 (3 subgenotype 3e and 2 subgenotype 3f strains) were found.

4. Discussion

The epidemiology of HEV infections among patients in Hungary is barely described. Here, we present a survey of the latest seroprevalence data about patients with symptoms and signs of acute hepatitis between 2018 and 2020 at the University of Szeged. We report an increase in IgG positive cases (18.4% in 2001–2006, while 31.0% in 2018–2020) compared to previously published results [9]. In a previous study, samples were collected between January 2001 and June 2006 in the same university hospital as our study was performed. However, a survey conducted in 2001 and 2006 focused only on the Infectious Disease Department; we selected patients from various wards because patients with underlying diseases were admitted to those wards where they cured their underlying conditions. We used earlier selection criteria in the case of patients chosen for our study [9]. We consider that the difference in IgG seropositivity can partly be explained by the increased number of serological testing using the most specific and sensitive commercially available ELISA kit (WANTAI Bio-Pharm, China) [12].

Several studies proved that the incidence of hepatitis E infection increased in many European countries during the last 10 years [6–8]. A comparison of the available findings showed that seroprevalence data in the East-Central European countries are quite similar. HEV seroprevalence reported for the general population in the neighboring countries is 17.8% in Slovakia (DRG Instruments), 15% in Serbia (in-house assay), and 14% in Romania (Mikrogen), 14.3% in Austria (Wantai) [13–15]. However, it is difficult to interpret these results and compare data obtained by using various kits and patient groups. Earlier studies have reported that the main route of HEV infection in developed countries is the consumption of raw or undercooked pork meat [16, 17]. In our survey, only 5 patients gave adequate information (pork, sheep, deer, and seafood) about food regarding as the possible source of HEV infections, other patients also ate meat before the development of acute HEV, but correct information (type of meat and time) was not available. Local statistical surveys have found that pork meat consumption per capita has increased in Hungary over the last decade, thus our higher seroprevalence result may partly be related to this tendency [18, 19].

International studies have reported that most HEV infections occurred in middle-aged and elderly males. In addition, acute hepatitis E is common in people who consume excessive amounts of alcohol, which is an important risk factor for hepatic fibrosis and steatosis [2, 20, 21]. Hungary has always belonged to an outstanding group of nations characterized by severe liver problems associated with excessive alcohol consumption [22]. Previous studies have found that liver damage caused by alcohol is prevalent among middle-aged and elderly men [23]. In our case, 6 (8.5%) out of 70 patients with acute HEV had chronic alcoholism, and 5 (7.1%) said they occasionally consume alcoholic drinks. We found that the number of males with acute HEV infection (n = 47, 67.1%) was significantly higher than females (n = 23, 32.9%) (P = 0.0013, RR = 2.213, CI: 1.367–3.589). We confirmed acute cases mostly above 40 (n = 67; 95.7%) with a mean age of 63 years. In Reuter's previous study, the mean age was also above middle age (53 years), and the ratio of males (n = 63, 54.3%) was higher than females (n = 53, 45.7%) [9]. Most anti-HEV IgG-positive cases were also above the age of 40 (n = 374, 87.2%). In addition, we found that cohorts above the age of 71 are the highest risk groups. Consequently, our results confirmed earlier findings that the most affected patients are the adult or elderly men (range 41–90 years), probably due to Hungary's previously mentioned drinking and eating habits. However, the comprehensive verification of these correlations deserves further comparative studies.

During our survey, infections occurred in all months, but two significant peaks were observed during the sampling period. These two

peaks coincide with two Hungarian slaughtering periods (smoked product is characteristic of winter, and barbecuing is typical in summer); the second peak also overlaps with harvesting fruits and vegetables [9, 24, 25]. Because of the lack of adequate information about the source of infection in our case, we need further investigation to identify the potential sources of HEV in Hungary. Similarly, Reuter et al. also found that acute cases were obtained mainly in April–June and December in Hungary [9]. The proportion of acute HEV infections confirmed by serological tests (70 (5.1%) out of 1383 patients) wtheseas lower compared to the previous survey (116 (9.6%) out of 1203 patients) [9]. That difference can be partly explained by the lower specificity of the anti-HEV IgM ELISA assay used in the previous study [9].

We detected HEV-specific PCR product in the case of 7 (10.9%) out of 64 patients with severe clinical symptoms and low IgG index value. Results of molecular assays were lower compared to former data (13 (24.5%) out of 53 patients) [9]. The explanation for that noticeable difference is probably the difficult detectability of HEV RNA, and in many cases, feces were submitted for PCR 2 weeks after the first admission of the patients and diagnosis of acute HEV infection. Although RNA can be detected in stool for up to 4–6 weeks, its amount can be very low, which reduces the success of the detection [4].

HEV is phylogenetically divided into eight genotypes (HEV-1–8). Among genotypes, HEV-1 and −2 infect humans and are transmitted by the fecal-oral route, while HEV-3 and −4 infections are mainly zoonoses, HEV-5–8 are detected in various animals. Inside HEV-3, three monophyletic clades can be distinguished group 1 (subtypes HEV-3e, f, and g), group 2 (HEV-3a, b, c, h, I, j, k, l, and m), HEV-3ra [25]. Phylogenetic studies have shown that genotype 3 is the most common genotype in developed countries. HEV subgenotypes 3e, 3f, 3c, are circulating in human and pig populations, and these strains occur mainly in Europe [1]. In Hungary, a previous study reported primarily detecting subgenotype 3e strains [9]. In our survey, we identified 3e and 3f strains, these strains were detected from hospitalized patients with jaundice and abdominal pain. All patients had underlying diseases, including diabetes, hypertension, chronic renal failure, and chronic myeloid leukemia.

5. Conclusion

Our survey confirmed the continuous presence of HEV infections in Hungary. We observed an increase in the number of IgG-positive cases among the studied patients. We think the increasing trend is partly due to increased testing by highly sensitive commercially available tests and the previously reported drinking and seasonal eating habits among middle-aged and elderly males. There seems to be a seasonal pattern of acute infection; however, further investigations are necessary to confirm this. The proportion of acute infections was noticeably lower compared to the previous study. Possibly, due to the differing specificity of ELISA assays or the slight difference between patient populations in these studies. The result of the molecular assay was also lower on account of the difficult detectability of HEV RNA and the delay in submitting stool for HEV RNA detection. The cases were typed as genotype 3 of the subtypes 3f and 3e, which are detected in hospitalized patients with jaundice and abdominal pain.

Ethical approval

Not required.

Authors' contribution

ÁBU performed enzyme immunoassays (EIA), molecular investigations, and data analysis and drafted the manuscript; MB performed statistical analysis; AM carried out EIA; ZT and EH supervised the acute hepatitis cases; KB critically reviewed the paper; GT designed the study, contributed to data interpretation and analysis; all the authors critically reviewed and finalized the manuscript.

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Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2022.105250.

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