

Szent István University
Postgraduate School of Veterinary Science

**The presence of Hepatitis E virus in domestic and wild animals in
Hungary; genetic analysis of the virus strains**

PhD dissertation thesis

Petra Forgách, DVM

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Supervisor and members of the supervisory board:

.....

Tamás Bakonyi, DVM, PhD
Szent István University, Faculty of Veterinary Science
Department of Microbiology and Infectious Diseases
supervisor

Miklós Rusvai, DVM, CSc
Szent István University, Faculty of Veterinary Science
Department of Pathology and Forensic Veterinary Medicine
member of supervisory board

György G. Szűcs, MD, CSc
Kuwait University, Faculty of Medicine
Department of Microbiology
member of supervisory board

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Petra Forgách, DVM

INTRODUCTION

Hepatitis E virus (HEV) is a non enveloped virus with 32-34 nm of diameter and icosahedral symmetry. The viral capsid contains a positive sense single stranded RNA. The viral genome is 7,5 kb in length and codes 3 partially overlapping open reading frames (ORF) (Emerson and Purcell, 2003). It belongs to the *Hepeviridae* family *Hepevirus* genus of virus families not assigned to an order (ICTV, 2010). The virus-variants detected in several countries are classified into 4 genogroups; viruses of the 3rd and 4th genogroup can infect both humans and animals.

The Hepatitis E virus spreads enterically, causing acute, self-limiting icteric hepatitis with the mortality of 1%. In several developing countries the human HEV infections are “endemic” (Anderson *et al.*, 2002; Schlauder *et al.*, 2003), where the seroprevalance is around 15% and large epidemics occur. The developed countries are considered being “non-endemic”, cases of clinical hepatitis E are sporadic, mainly imported infections occur; however some autochthonous cases have been published already. The infections in such cases are usually originate from contact with infected animals (Yazaki *et al.*, 2003; Widdowson *et al.*, 2003; Wichmann *et al.*, 2008). Animal reservoirs are considered to play an important role in the maintenance of the virus and in the spread of HEV to humans. HEV-induced sero-conversion was described in several species, however clinical hepatitis in animals has not been observed to date. Besides genetic and epidemiological studies, the zoonotic nature of the virus was proven by experimental infections. HEV infections of animals remain subclinical. Besides domestic swine, wild boar and several deer and roe deer species were found to be the main reservoir hosts of HEV (Meng *et al.*, 2005).

The phylogenetic analyses indicated close genetic relationships between hepatitis E viruses from human and animal origins, which further support the theories about the zoonotic character of the virus. The reservoir role of animals is suspected; the HEV infection of animals has both food-hygiene and occupational health importance (Galiana *et al.*, 2008).

THE AIMS OF THE STUDY

Among viruses causing enteric infections in animals the zoonotic potential of rotaviruses, picobirnaviruses and caliciviruses is presumed on the basis of genetic similarities (van der Poel *et al.*, 2000; Bányai *et al.*, 2005; 2008; 2009, Matthijssens *et al.*, 2008; Martella *et al.*, 2010). The zoonotic character and food-borne spread of HEV is suggested by previous investigations (Meng *et al.*, 1998; Yazaki *et al.*, 2003; Tamada *et al.*, 2004). The aim of our study was to detect HEV nucleic acid by reverse transcription-polymerase chain reaction (RT-PCR) in samples of animal origin collected in Hungary. We wanted to define the range of animal species – particularly food-producing animals - that have role in the spread of the virus. By selective sampling at swine farms, data were collected on the level of infection in different age groups. The spread of the infection within the farms, the level of infection at the slaughtering age group and the way of infection was also analysed. Samples of rodents (mice, rats) at swine farms and specimen from small mammals (common shrews, hamsters) from natural environment were collected and analysed to assess the role of these animals in the epidemiology of HEV.

During a scholarship program at the University of Veterinary Science archived human sera samples were investigated in order to compare two different serological assays and survey the presence of HEV infection in Austria as well as to measure the occupational health account of HEV infection among swine-related occupational groups.

Phylogenetic analyses were performed to infer the genetic relatedness of the detected viruses as well as to collect information on the genetic evidences of the zoonotic potential of HEV. By the determination of the nearly complete sequence of a Hungarian HEV strain the evolution of the virus and the possibilities in the genotyping was investigated.

MATERIALS AND METHODS

In the survey on the occurrence of HEV infection in animals, we collected samples in which the viral nucleic acid can be detected with high confidence. All together 717 samples were investigated that were collected from the main reservoirs (domestic swine, wild boar, roe deer and red deer) as well as domestic ruminants (cattle, sheep, goat) and small mammalians (mouse, rat, common shrew and hamster). Liver and faeces samples of swine were collected during necropsy. Faeces samples were also collected from living animals kept at swine farms. Wild boar, roe deer and red deer liver samples were collected in a game processing slaughterhouse. Faeces samples of domestic ruminants originated from different farms were also investigated. Feed, rat and mouse samples were collected in HEV-infected swine farms, while common shrew and hamster samples were collected from natural habitats. For serological investigations archived human sera samples were used that were collected in the area of Graz (Austria) from people with frequent contact with swine.

During the preparation of the RT-PCR reactions, the samples were homogenized and viral RNA was extracted. The homogenized samples and viral RNA was stored at -80 °C. The samples were tested first with a primer-pair, which anneal within the ORF2 region, and positive results were confirmed by a second RT-PCR with primers annealing the ORF1 (Schlauder *et al.*, 1999; van der Poel *et al.*, 2001). To determine the nearly complete genome sequence, apart from the “diagnostic” primer pairs 15 additional primer pairs were designed, amplifying overlapping products in different size. For the RNA-extraction and RT-PCR the QIAamp Viral RNA and OneStep RT-PCR kits (Qiagen, Düsseldorf, Germany) were used according to the manufacturer’s instructions.

Following the RT-PCRs, 5µl of the amplification products were electrophoresed and the bands were visualized by UV transillumination. Specific amplification products were excised from the low melting agarose gel, and DNA was extracted using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The nucleotide sequences of selected amplification products were determined in fluorescence based direct sequencing reactions, in two directions, using the

previously described primers. The sequences were identified by the Basic Local Alignment Search Tool (BLAST, <http://www.ncbi.nlm.nih.gov/BLAST>) search against the gene bank databases. The sequence corrections and multiple alignments were made with the help of BioEdit 4.7.8 and Clustal X programs, the analyses were performed using the Phylogeny Inference Program Package (PHYLIP) version 3.6b. The sequences determined in this study were compared to HEV genotypes deposited in the GenBank database. Bootstrap resampling analyses of 1000 replicates were generated with the seqboot program to test the stability of the trees. Neighbor-Joining distance matrices were generated by the dnadist and Fitch programs, using a transition/transversion ratio of 2.0. Phylogenetic trees were delineated with the help of the TreeView program. Graphical analysis of the similarity of different HEV strains were done using SimPlot 3.5.1. software.

The *recomBlot* HEV IgG/IgM[®] (Mikrogen, Neuried, Germany), was used in the serological investigations. Both HEV IgM and IgG antibodies were determined in all sera. Test evaluation was done as described in the manual of the kit. Samples found positive or weak positive in the immunoblot investigation as well as some negative samples applied as controls were tested by ELISA. The investigation was done using *recomWell* HEV IgG[®] and *recomWell* HEV IgM[®] ELISA kits. The optical density (OD) values were visualized by Genesis Lite 3.03 software. The test procedure and evaluation was done as described in the manual of the kit.

The IgM positive sera samples were subjected to HEV-specific RT-PCR assays to detect viral nucleic acid. RNA extraction, RT-PCR using the diagnostic primer pairs, the visualization of PCR products and sequencing were done as described previously.

RESULTS

HEV RNA was detected in 62 (25 %) of the investigated 251 domesticated swine: 52 (21%) of 248 faeces and in 14 (31%) out of 45 liver samples were found positive. The samples were collected in 41 Hungarian pig farms, and 16 (39%) of these farms were found to have HEV-infected swine. Information on the age of 204 domestic pigs was available. These animals were classified into four age groups: 1-4 week old piglets, 5-10 week old pigs, 11-16 week old pigs, and swine over 17 weeks. The frequencies of the HEV positive samples of these groups were 9%, 27%, 36%, and 10% respectively (Figure 1). In 42 animals, faeces and liver samples were investigated simultaneously. Differences were not found in the positivity of the investigated faeces and liver samples.

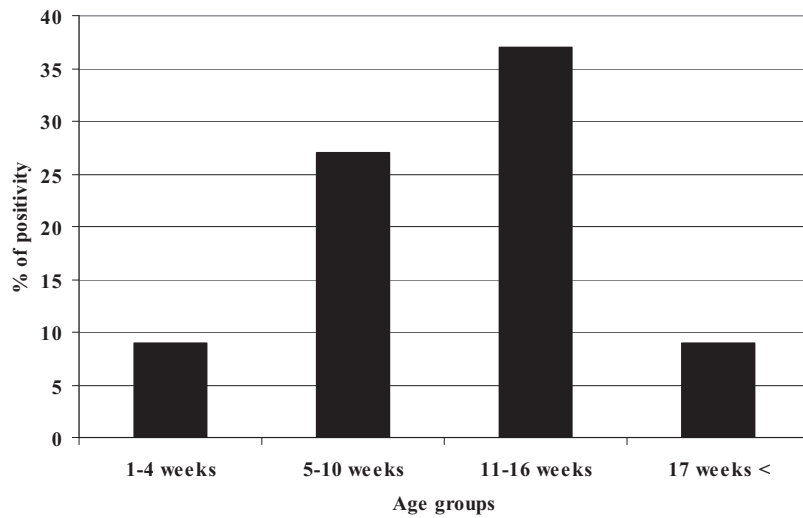


Figure 1. Prevalence of HEV infection in different age groups of domesticated pigs.

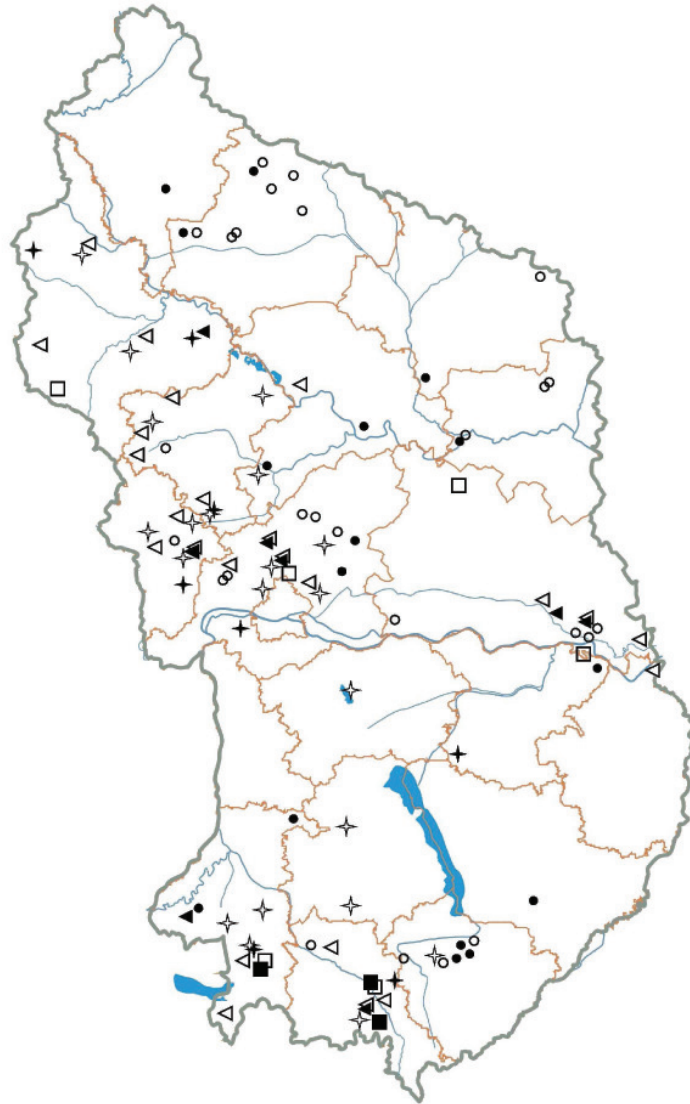


Figure 2. Geographical origin of the HEV-positive animal samples. Dots indicate the domestic swine farms, triangles are wild boars, stars are roe deer and squares are red deer samples. Positive samples are marked with filled; negative samples are marked with empty shapes.

The cattle faeces samples, the rat, mice, hamster and shrew samples, as well as the faeces samples of wild ruminants and wild boar did not contain HEV RNA. Eight out of 75 (9%) wild boar, 9 out of 41 (22%) roe deer and 3 out of 30 (10%) red deer liver samples contained HEV RNA. The geographical origins of the positive samples are demonstrated in (Figure 2).

By the results of the immunoblot assays the anti-HEV seropositivity in the swine-related occupational groups (swine farmers, swine slaughterhouse-workers) was higher, among hunters and veterinarians was around the mean and in the control groups (poultry slaughterhouse workers and townspeople) the anti-HEV seroconversion was lower. The results of the ELISA test differ from these findings: the seropositivity did not correlate with the frequency of the contact with swine.

Twenty nine amplification products of the partial ORF2 region and five selected products of the partial ORF1 region of the HEV-positive animal samples were sequenced. All sequences showed the highest similarity to HEV sequences. The phylogenetic analysis of the partial ORF2 region revealed that the viruses detected in Hungarian animal samples belong to 3 subgroups the 3rd genogroup of HEV showing high similarity to HEV sequences of human and animal origin detected in other countries (Figure 3). Although the variability of the nucleotide sequence of the virus is quite high, the analysis of the deduced amino acid sequences indicates that the nucleotide-differences are mainly silent mutations; only in a few cases there are single amino-acid substitutions in the sequences.

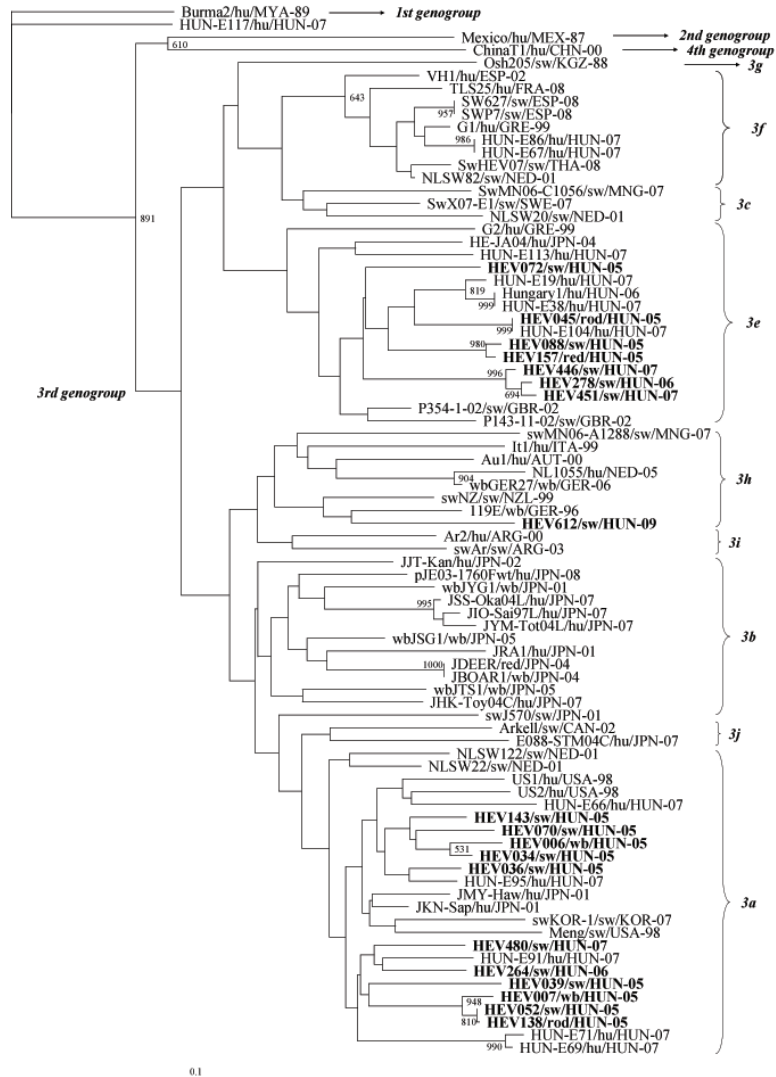


Figure 3. Neighbor-joining phylogenetic tree based on the partial ORF2 region of HEV genotypes. The viruses are marked as follows: strain/host (Sw: domestic swine, Wb: wild boar, Hu: human, Red: red deer, Rod: roe deer)/country (three letters sign) – year of detection. Sequences determined in this study are indicated in bold.

In the case of a selected sample (HEV072) a 7189 long continuous nucleotide sequence was determined. At nucleotide level the sequence of the Hungarian virus 80-89% similarity to the 3rd genogroup viruses of both animal and human origin detected in several countries. At amino acid level the similarity of all 3 ORFs is above 90%: 90-94% at ORF1, 96-98% at ORF2 and 90-97% at ORF3 regions. By SimPlot analysis 2 characteristic regions were found. Between the 2125-2500 nt positions [polyproline hinge (PPH) or hypervariable (HVR) region] the sequences of the viruses belonging to 3rd genogroup are very different (the similarity at this region is 14-76%). Between the 5250-5500 nt position (putative capsid protein, PCP region) the sequences of the 3rd genogroup viruses are very similar (the similarity at this region is 93-98%) (Figure 4.). At amino acid level the similarity among the 3rd genogroup viruses at the HVR region is 64-80% while at the PCP region is 91-99%.

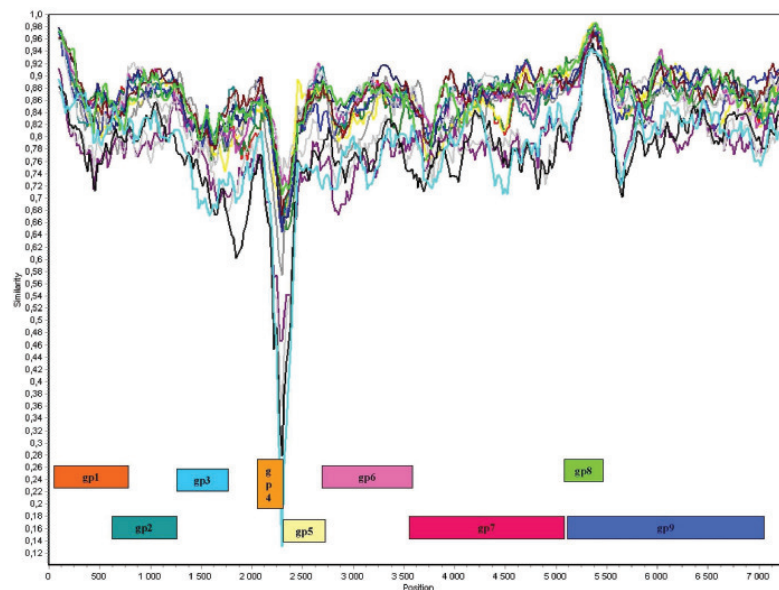


Figure 4. SimPlot analysis of 3rd genogroup viruses. The different sequences are marked with different colours.

DISCUSSION

The occurrence of HEV infection amongst domestic swine appears to be moderate comparing to the results of surveys done in other countries (Fernandez-Barredo *et al.*, 2006 and 2007; Rutjes *et al.*, 2007; Di Bartolo *et al.*, 2008). The purpose of investigating HEV in different pig age groups was to determine the course of infection in HEV infected pig farms. The source of the infection is usually the infected sow, or after weaning, infected pigs with which the susceptible animals are grouped together. The infection can spread by the contact with the faeces containing virus particles, or by the faecal-contaminated feed. In Hungary, the highest (37%) HEV positivity was observed amongst the eleven to sixteen week old pigs, and the presence of HEV RNA was detected in 27% of samples collected in the five to ten week old age group. Low virus-shedding was detected among the suckling pigs (9%). Infected pigs could be found in the finishing age groups (9%) too, leading to the possibility of slaughter and consumption of HEV infected pig meat. The age distribution of the HEV infection amongst domestic pigs in Hungary is in line with the results detected in other countries.

Nevertheless, our results did not support the theories on the higher risk of HEV infection in swine-related occupational groups. Other studies found higher seroprevalance among people with frequent contact with swine comparing to control groups. By the comparison of two different serological assays we found that the applied immunoblot test, – probably because of its subjective evaluation method – is not reliable for diagnostic purposes. Storage conditions and time could also have negative effect on the quality of the sera samples and in consequence on the results of serological tests

In routine diagnostic investigation of living and dead swine, both liver and faeces sample seems to be suitable for the reliable detection of HEV infection.

The presence of HEV was detected also in the wild ungulate populations in Hungary. Comparing to the results of other studies, the prevalence of HEV among wild boars in Hungary is moderate (Michitaka *et al.*, 2007; Martelli *et al.*, 2008; de Deus *et al.*, 2008; Kaba *et al.*, 2009; Schielke *et al.*, 2009). The HEV infection of wild ruminants is poorly

investigated so far. Comparing to results of a study done in Japan (Takahashi *et al.*, 2004), the prevalence of HEV infection in the Hungarian roe deer and red deer population is relatively high. The spread of hepatitis E virus between wild animals, the course of infection in different age groups and species, and the subsistence of the HEV infection in natural habitats are subjects of further investigations.

The role of small mammals in the maintenance of HEV infection at swine farms, and in natural habitats was suspected, because these animals are susceptible for HEV infection. However the viruses detected in rat and rabbit samples are genetically differ from viruses of human and swine origin (Zhao *et al.*, 2009; Johne *et al.*, 2010). Up to now natural infection of small mammals by human and swine HEV strains was not proved. In our investigations HEV nucleic acid was not detected in rat, mouse, hamster and shrew samples, therefore the veterinary and public health aspects of the reservoir role of these animals should be clarified by further investigations.

The HEV strains detected in animal samples collected in Hungary belongs to 3 subgroups of the 3rd genogroup of HEV. Genetic similarities of different virus strains are independent from the geographical distance of the place of detection. Viruses found in swine farms near to each other could differ more than viruses detected at places being far from each other or viruses detected in different species. The phylogenetic analyses show that within 1.5 years genetically different HEV strains can circulate simultaneously on the same farm. The close genetic correlation between the animal strains found in Hungary and human HEV strains from different countries is in line with previous publications suggesting the zoonotic potential of HEV.

In the investigation of the nearly complete genome sequence of a selected Hungarian strain several genome regions were analysed. Among the 3 ORFs the ORF2, which codes the structural proteins, was found to be the most conservative region. The high variability of the non-structural proteins coding ORF1 can be explained by the hypervariable region of the polyproline hinge domain. By the results of the analysis, besides the complete genome sequence, the ORF1 region and the sequence produced by the diagnostic ORF2s1-ORF2a1 were found to be suitable for genotyping of

the detected virus strains. However, for the reliable results, sequences of several virus strains belonging to different subgroups is needed to be involved into the investigations. In the case of the 3rd genogroup viruses, the HVR region of the genome can be used for epidemiological investigations, although further investigations are needed to reveal the reasons of the high variability of the region, as well as the conditions and epidemiological consequences of the insertions/deletions in the genome sequence. The most conserved part of the genome was found to be inappropriate for the design of universal primers but this region may be suitable for probes in *in situ* hybridizations.

NEW SCIENTIFIC RESULTS

1. The survey on the presence of HEV in animal samples described the presence of the virus in Hungarian domestic swine populations. The level of the infection in swine farms was also established (39%).

2. Food-hygiene risk of the infection of slaughtering pigs was revealed. By the results of simultaneously investigated faeces and liver, both samples were found to be suitable for the detection of HEV infection.

3. The presence of HEV in wild ungulates (wild boar, red deer, roe deer) was detected for the first time in Hungary. By the geographical origin of the positive samples the HEV infection of animals, similarly to human cases, can be detected in the whole territory of the country.

4. Phylogenetic investigations revealed that the virus strains detected in animals belonged to 3 subgroups of the 3rd genogroup of HEV. High genetic diversity was observed among the detected virus strains as well as similarities to strains of human and animal origin detected in Hungary and other countries.

5. By the determination and analysis of the nearly complete sequence of a selected Hungarian HEV strain, the genetic variability of the different genomic regions of the HEV was characterized. Two typical genome regions were found which accurately represent the phylogenetic relations of the different virus strains.

6. By the analysis of the nearly complete genome sequence and 7 different parts of the genome, regions suitable of the genotyping were determined.

7. By the comparison of two serological assays we assessed that the subjective evaluation of the tests and the quality of sera samples can negatively affect the reliability of the results.

The results of our studies can serve as basis of further food-hygiene, veterinary, epidemiological and public health investigations.

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