Morphological and immunophenotypic characteristics of the liver of swine naturally infected with hepatitis E virus

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Summary
Hepatitis E virus (HEV), the zoonotic agent of infectious hepatitis, is present in swine farms in different geographical areas. Little is known about the mechanism of liver damage and type of local immune response by HEV in swine. Therefore, the aim of this study was to determine the morphological and immunophenotypic characteristics of hepatic lesions caused by hepatitis E virus in naturally infected swine. In this study, liver samples of 12 slaughtered 10 weeks old pigs which were RT-PCR positive for HEV RNA in rectal swab samples have been used. Livers were macroscopically examined and samples were taken for histopathological, immunohistochemical (CD3, CD79α and TGF-β1), semiquantitative, morphometric analysis, RT-nested-PCR, PCR and bacteriological analysis. Microscopically, mild and moderate multifocal lymphoplasmacytic hepatitis was observed. Apoptotic bodies were observed as areas of focal eosinophilic condensation in the cytoplasm of 33.33% liver samples, while in 16.67% liver samples portal fibrosis was detected. Immunohistochemically, portal and lobular lymphocytes in the mononuclear liver infiltrate were predominantly CD3+ T cells (234.80 ± 79.98). An intense TGF-β1 positive reaction was observed within the mononuclear cell infiltrate as well as polymorphonuclear cells in liver samples with apoptosis of hepatocytes. In all 12 tested liver samples HEV RNA was detected by RT-nested-PCR. HEV is noncytopathic, and this finding provides further evidence for an immune mediated pathogenesis in hepatitis E virus infection in swine. Also, the role of CD3+ cells in hepatocyte damage is clearly demonstrated.

Introduction

Hepatitis E virus, the zoonotic agent of infectious hepatitis, is present in swine farms in a number of geographical areas (Aprea *et al.* 2018). Different species of domestic and wild animals have been reported to have anti-HEV antibodies (Okamoto 2007, Roić *et al.* 2018), and/or to be infected with viruses closely related to HEV strains infecting humans (Tei *et al.* 2003, Banks *et al.* 2004). Swine infected with HEV are asymptomatic. A number of proves have demonstrated the zoonotic nature of hepatitis E virus. Swine and human HEV strains are genetically related suggesting both a zoonotic and a possible foodborne transmission (Meng 2003, Di Bartolo *et al.* 2008, Caruso *et al.* 2017, Bansal *et al.* 2017).

Usually, there are no gross lesions in the liver of HEV infected pigs. Occasionally mild hepatic enlargement and scattered yellowish discoloration foci can be found in some samples (Lee *et al.* 2007).

Microscopically, naturally and experimentally HEV-infected pigs showed evidence of acute hepatitis characterized by mild to moderate multifocal and periportal lymphoplasmacytic hepatitis, with mild focal hepatocellular necrosis (Lee *et al.* 2007, Halbur *et al.* 2001, de Deus *et al.* 2008, Lee *et al.* 2008).

Viral infection causes host cell injury either directly, through the action of an infectious agent, indirectly, as a consequence of the antiviral host immune response or through the cumulative effects of both direct and indirect damage (Srivastava *et al.* 2007). While HEV is effectively cleared by a strong immune response, hepatic damage of a variable severity is a common consequence of cytotoxicity. Conversely, an inadequate immune response results in viral persistence, although with little liver damage (Srivastava *et al.* 2007). Studying the direct cytopathic effect of HEV has proven difficult, due to the inability to efficiently culture the virus *in vitro* (Srivastava *et al.* 2007). An aggressive immune response leads to effective viral clearance that is accompanied by a variable degree of hepatic damage (Srivastava *et al.* 2007). On the other hand, an inadequate cytotoxic response results in viral persistence, albeit with little liver damage (Srivastava *et al.* 2007). In any case, the mechanisms which regulate the intensity of the immune response have a key role in the viral infection defense.

It has been hypothesized that liver damage induced by HEV infection in humans may be due to the immune response to the invading virus and may not be a direct cause of viral replication in the hepatocytes (Purcell 1996). Nevertheless, some authors emphasize that the immune-mediated liver injury by lymphocytes might be mainly involved in the pathogenesis of hepatitis E, but liver injury induced directly by HEV could not be excluded (Zhao *et al.* 2001).

In cases of acute fulminant human hepatitis E, the lymphocyte infiltrate consisted predominantly of CD3+ T cells. These cells contained a predominant cytotoxic (CD8+) cell subpopulation in 81.8% of cases with hepatitis E infection (Agrawal *et al.* 2012). The cellular composition of the liver inflammatory infiltrate was different in patients with B and C hepatitis where T helper lymphocytes comprised 50-60% of the inflammatory infiltrate. Approximately 25% were T cytotoxic lymphocytes; B lymphocytes comprised 15% of the inflammatory infiltrate and other cells, including natural killer cells (NK), 10% in total (Waleska-Zielecka *et al.* 2008).

Immunohistochemical findings of the liver biopsy samples of HEV infected patients clearly demonstrate the role of CD8+ T cells and natural killer cells in hepatocyte damage and disease pathogenesis. The virus is noncytopathic, and therefore, liver injury may be attributed to immune-mediated damage by cytotoxic T cells and natural killer cells (Prabhu *et al.* 2011).

Although many studies have described histological alterations of the swine liver, little is known about the mechanism of cell damage or the type of local immune response by the host cells to HEV. Therefore, the aim of this study was to determine the immunophenotypic characteristic of immune cells in swine liver naturally infected with HEV.

Materials and methods

Sampling

Rectal swabs were taken from 153 weaned pigs from the same farm and tested for the presence of HEV RNA by RT-nested-PCR. Thereafter, 12 positive pigs were slaughtered in the slaughterhouse and livers were macroscopically examined and liver samples were taken for histopathological, immunohistochemical, semiquantitative, morphometric analysis, RT-nested-PCR and bacteriological analysis. Additionally, spleen samples were taken for PCR analysis for swine Torque Teno Virus (TTV) and Porcine circovirus 2 (PCV2), whereas lung samples were used for RT-PCR for Porcine Reproductive and Respiratory Syndrome virus (PRRSV) detection. The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals (Ethics Committee of Faculty of Veterinary Medicine, University of Belgrade, 01.06.2011, No. 01-624). Liver samples from 10 pigs which were negative for HEV RNA were used as negative controls. Previously, swine sera from both groups were tested negative for PCV-2 and PRRSV antibodies.

Histopathological and immunohistochemical studies

Liver samples for histopathological and immunohistochemical studies were fixed in 10% buffered formalin and after standard processing in an automated tissue processor (dehydration, illumination and impregnation), cast in paraffin blocks. The paraffin sections 4 µm thick were stained with hematoxylin and eosin (HE) and Masson trichrome method (MTH) for light microscopic examination. Previously, fresh liver samples were fixed in fixative consisting of 9 parts by volume of absolute ethyl alcohol to one part of 40% formaldehyde neutralized with $MgCO_3$ and paraffin sections were stained by Best's carmine technic for excluding accumulation of glycogen in hepatocytes. Additionaly, cryostat sections of the liver samples

Table I. *Primary antibodies used for immunohistochemistry.*

Antibodies	Source	Dilution	Antigen retrieval	Incubation
CD3	DAKO A0452	1/50	microwave, 560W, 21 minutes in citrate buffer	1 h, in humid chamber at 22 ± 3 °C
CD79	DAKO M7051	1/50	microwave, 560W, 21 minutes in citrate buffer	1 h, in humid chamber at 22 ± 3 °C
TGF-β1 (v)	SANTA CRUZ BIOTECHNOLOGY, sc-146	1/10	proteinase K treatment for 40 minutes at 22 ± 3 °C	Overnight, in humid chamber at 4 °C

were stained by Sudan III for excluding potentially fatty liver degeneration, as well.

Immunohistochemical procedure

A three-step indirect immunohistochemical technique was performed on 4 µm formalin-fixed and paraffin embedded sections. After antigen retrieval (Table I) the sections were then treated with methanol containing 0.3% hydrogen peroxide for 15 minutes at 22 ± 3 °C in order to inactivate the endogenous peroxidase. Non-specific binding of secondary antibodies was minimized by incubating with 50% normal goat serum in PBS for 20 minutes. Sections were incubated with appropriate primary antibodies (Table I) diluted in PBS. Three primary antibodies were applied: rabbit-anti-human CD3 (pan T-cell marker), mouse-anti-human CD79α (B-cell marker) and rabbit polyclonal antibody TGFβ1 (v). All rinsing procedures and serum dilutions were done in PBS (pH 7.2-7.4). The detection kit was DAKO Cytomation LSAB2 System-HRP, Rabbit/mouse (DAKO, K0675). Positive reactions were visualized by applying DAB+ (DAKO, K3468) for 5 to 10 minutes. Counterstaining was performed using Mayer haematoxylin for 2 seconds. Aqueous medium glycergel (DAKO, C563) was used on stained sections for mounting. Liver sections not treated with the primary antibody were used as negative controls. As positive controls for immunohistochemistry (IHC) reactive lymph nodes of swine were used.

Semiquantitative analysis

Semiquantitative analysis was conducted according to criteria which were used for assessment of distribution and density of lymphoplasmacytic infiltrates in swine livers experimentally infected with HEV (Halbur *et al.* 2001). Score for severity of lymphoplasmacytic hepatic lesions are shown in Table II.

Morphometric analysis

Immunopositivity was quantified by counting the total number of lobular and portal CD3 and CD79α immunopositive cells in 25 random selected fields at 40× magnification. The area of the liver specimen was calculated using image analysis software (Olympus Cell B), and the cell count per mm^2 was calculated. This was compared between the observed cases and controls.

Digital images were taken by microscope Olympus BX51 with digital camera Olympus Color View III.

RNA extraction and HEV RT-nested-PCR and PCR

Rectal swabs were prepared by immersion into sterile PBS and vigorous vortexing. Organ samples were homogenized in sterile PBS in final dilution 1:10. After homogenization, the suspensions were centrifuged for 10 min at 2000 rpm. The supernatant was used for nucleic acid extractions.

DNA was isolated from liver samples by using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the recommended tissue protocol.

Viral RNA from rectal swabs and liver samples was extracted by using QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

RT-PCR and nested-PCR were carried out in 50 µl reactions using OneStep RT-PCR Kit and HotStar Taq MasterMix Kit (Qiagen, Hilden, Germany), respectively.

Two degenerate primer sets were selected for the amplification of ORF2 region. Primers used for the first round were HEVORF2con-s1 - 5'GACAGAATTRATTTCGTCGGCTGG - 3' and HEVORF2con-a1-5'CTTGTTCRTGYTGGTTRTCATAATC - 3'. For the nested-PCR, primers HEVORF2con-s2 - 5'GTYGTCTCRGCCAATGGCGAGC - 3' and

Table II. *Criteria for semiquantitative analysis.*

Score for severity of lymphoplasmacytic hepatic lesions	Description
0	No inflammation
1	1 to 2 focal lymphoplasmacytic infiltrates/ 10 hepatic lobules
2	2 to 5 focal lymphoplasmacytic infiltrates/ 10 hepatic lobules
3	6 to 10 focal lymphoplasmacytic infiltrates/ 10 hepatic lobules
4	> 10 focal lymphoplasmacytic infiltrates/ 10 hepatic lobules

HEVORF2con-a2-5'GTTCRTGYTGGTTRTCATAATCCTG - 3' were used, yielding a final product of 145 bp (Erker *et al.* 1999).

Both reactions were accomplished following the thermal profiles previously described (Di Bartolo *et al.* 2008).

For TTV, PCV2 and PRRS detection, we used the previously described primers and protocols (Oleksiewicz *et al.* 1998, Lukač *et al.* 2016, Kekarainen *et al.* 2006).

Bacteriological analysis

For isolation of pathogenic bacteria (*Staphylococcus aureus, Trueperella pyogenes, Escherichia coli, Klebsiella* spp., *Proteus* spp., *Salmonella* spp., *Clostridium novyi, Listeria* spp.) from the liver samples, pre-enrichment was performed in various selective broths followed by plating on selective media as per the method described by Cruickshank and colleagues (Cruickshank *et al.* 1975). Identification of the bacteria and biochemical tests were done according to the standard procedures given by Cowan and Steel (1993).

Statistical analysis

After counting the total number of CD3 and CD79α immunopositive cells, the average number ($\overline{X}$), standard deviation (SD), minimal and maximal value (X_{min}, X_{max}) were calculated for each examined group. The cell counts among the groups were compared using ANOVA test. The significance of differences was determined by the level of significance of 5% and 1%. Statistical comparison of score for severity of lymphoplasmacytic hepatic lesions between infected and control groups has been done with Mann-Whitney test. Statistical analysis was carried out using GraphPad Prism (ver. 6.01).

Results

HEV RT-nested-PCR and PCR

In all 12 liver samples tested, HEV RNA was detected by RT-nested-PCR. All tested samples were negative for the presence of nucleic acid of PCV2, PRRS and TTV.

Bacteriological results

All liver samples were tested negative for the presence of pathogenic aerobic and anaerobic bacteria.

Macroscopic findings

Mild liver enlargement with blunt edges was demonstrated by macroscopic examination. Additionally, mildly to moderately enlarged hepatic lymph nodes in 6 (50%) out of 12 examined livers were also observed. In control group of pigs those findings were not noted. A markedly dilated gallbladder was observed in four cases (33.33%) among infected pigs and in two cases among pigs from control group (20%). The gallbladder was enlarged, protruding over the edge of the liver and filled with a large amount of bile.

Histopathological findings

Mild to moderate multifocal lymphoplasmacytic hepatitis in the portal tracts and/or irregularly distributed in the liver parenchyma was observed (Figures 1, a and b). The infiltrate comprised predominantly mononuclear cells, usually observed close to the area of necrosis of the hepatocytes. In all of the 12 examined samples a mild and moderate mononuclear infiltrate was localized in the portal tract. In 10 out of the 12 examined cases (83.33%) a multifocal mononuclear infiltrate and hydropic degeneration were observed in the liver parenchyma. Intracellular edema was present randomly, but with a higher prevalence in the centrilobular area. Activated Kupffer cells were detected in 7 out of 12 cases (58.33%). Apoptotic bodies which represent areas of focal eosinophilic condensation in the cytoplasm were observed in 4 out of 12 (33.33%) samples (Figure 1c). Apoptotic bodies were frequently observed in all three lobule areas, but they were more frequently found in the centrilobular and midzonal area. In 2 out of 12 cases, severe hepatocyte necrosis was observed mainly in the centrilobular area. In the remaining 10 cases, focal points of necrosis of the hepatocytes were observed mainly in the centrilobular and midzonal area and less frequently in the periportal areas. These focal points of necrosis were visualized as areas of parenchyma showing a complete destruction of cells and its transformation in cellular debris with lymphocytic and occasionally neutrophilic infiltrate. The degree of inflammatory cell infiltrate was significantly increased in the necrotic and degenerated liver parenchyma.

Borders between the portal tracts and lobuli were intact, and the lobular liver architecture was intact.

In uninfected control livers of pigs, microscopically there were no lesions in four samples. In six samples, very mild focal lymphoplasmacytic infiltrates were observed. In this group there were no signs of hepatocyte degeneration, apoptosis and/or necrosis.

In addition to the accumulation of large amounts of connective tissue in the portal tracts, around the dilated bile ducts, proliferative epithelium surrounded by multiple smaller bile ductules were observed (Figure 1d). The blood vessels in the portal

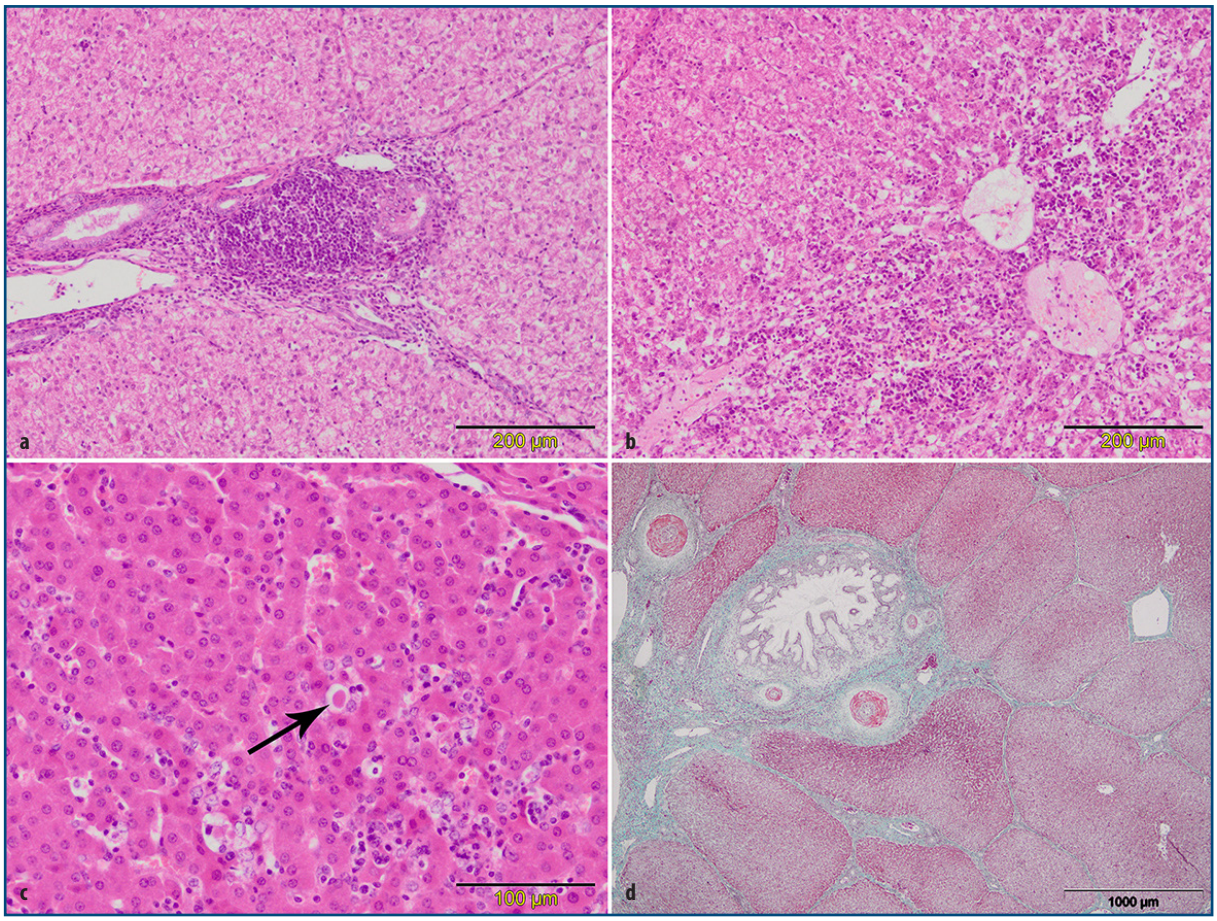

Figure 1. *Microscopic changes in the liver of pigs under study.* **a**. Severe portal mononuclear infiltrate. **b**. Mononuclear inflammatory cells observed closed to the area of necrosis of the hepatocytes. **c**. Councilman apoptotic bodies, Arrow shows Councilman apoptotic body. **d**. Portal fibrosis, HE.

tracts had a thickened tunica media with clearly visible amplified smooth-muscle cells. This finding is suggesting a chronic liver injury and fits into the portal liver fibrosis pattern.

Semiquantitative analysis results

Liver samples of infected pigs had the most frequent lymphoplasmacytic hepatic lesions severity score 1 (41.67%), following by score 2 in 3 (25%) tested samples. The most severe score 4 was as prevailing as score 3, both found in 2 cases (16.67%), respectively. In the tested liver samples of control pigs, the most common score was 0 (40%), and score 1, as well as score 2 were found in 3 cases (30%), respectively. A Mann-Whitney test indicated that the infected pigs' livers (mean rank = 14.25) were rated more severe than the control group (mean rank = 8.20), $Z = -2.261$, $p = 0.014$. The difference between tested groups is statistically significant ($p < 0.05$).

Immunohistochemistry

Immunohistochemically, portal and lobular lymphocytes in the mononuclear liver infiltrate were predominantly CD3+ T cells (Figure 2a). Immunopositivity was clearly demonstrated in the area of the plasma cell membrane. A smaller number of CD79α+ cells was detected in swine liver (Figure 2b). These cells were individually located within the regions of T-cell infiltration. TGF-β1 positive cells were detected within mononuclear cells infiltrate, as well as polymorphonuclear cells located in the liver parenchyma (Figure 2c). The intense positive reaction to TGF-β1 showed inflammatory cells in liver samples with hepatocyte apoptosis.

Morphometric analysis

Number of CD3 and CD79α positive cells was higher in infected pigs than in control pigs ($p < 0.05$, $p < 0.01$) (Table III).

Discussion

There is a lot of literature data on the prevalence of HEV infection in pigs in different countries throughout Europe. In addition, numerous studies

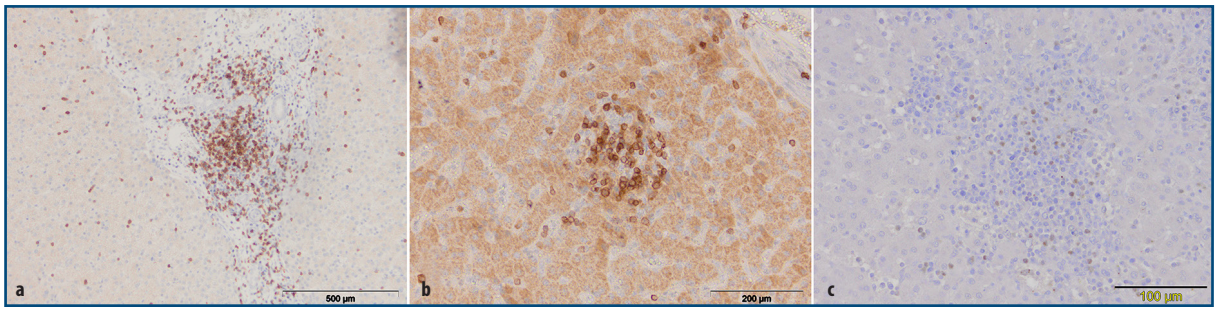

Figure 2. *Immunohistochemical characteristics of the liver of swine.* **a**. CD3 positive cells. **b**. CD79α positive cells. **c**. TGF-β1 positive cells, LSAB2.

Table III. *Number of cells expressed per mm² (n/mm²).* All values are given as mean values ± standard deviation.

Cell marker	Infected group $\overline{X} \pm SD\ (X_{min} - X_{max})$ (n = 12)	Control group $\overline{X} \pm SD\ (X_{min} - X_{max})$ (n = 10)	p value
CD3	234.80 ± 79.98 (166-350)	183 ± 8.49 (177-189)	$p < 0.05$
CD79	26.00 ± 17.71 (10-49)	3.00 ± 1.41 (2-4)	$p < 0.01$

$p < 0.01$ = statistically significant at the level of 99%;
$p < 0.05$ = statistically significant at the level of 95%.

are based on the molecular epizootiology and genotyping of hepatitis E virus (Di Bartolo *et al.* 2008, Vasickova *et al.* 2009, Forgach *et al.* 2010). Some reports were based on pathomorphological investigations in hepatitis E (Lee *et al.* 2007, Lee *et al.* 2008, de Deus *et al.* 2007), whereas studies on the local immune response in HEV infected animals have not been reported previously. Due to these reasons, the current investigations were conducted.

Macroscopic examination of the liver of pigs naturally infected with hepatitis E revealed a slight increase in volume which is consistent with the descriptions of other authors (Lee *et al.* 2007). In assessing this finding, one should be very cautious with regard to the enlargement of the liver, as it may be the result of increased amounts of blood and other infectious agents (PCV2, TTV, PRRS and bacteria such as *Clostridium novyi, Listeria* spp., *Trueperella pyogenes, Leptospira* spp., *Salmonella* spp.). However, in the current study, these agents were excluded by molecular methods and bacteriological analysis. The markedly dilated gallbladder cannot be linked to the HEV infection, due to the fact that such a pathological change usually occurs in the case of bile duct obstruction and in cases where the animals do not consume food and there are no stimuli for the secretion of bile. In this case it can be cosequence of feed restriction before slaughtering, which is usual practice. The observed increase in volume in hepatic lymph nodes in 50% cases might be due to reactive hyperplasia which is also described by Bouwknegt and colleagues (Bouwknegt *et al.* 2009).

Microscopically, naturally and experimentally HEV-infected pigs show evidence of acute hepatitis characterized by mild to moderate multifocal and periportal lymphoplasmacytic hepatitis, with mild focal hepatocellular necrosis (Lee *et al.* 2007, Halbur *et al.* 2001, Lee *et al.* 2008, de Deus *et al.* 2007), which was confirmed in our investigation with naturally infected pigs.

Furthermore, in all 12 samples (100%) the infiltrate was localized in the portal area of the liver, and in 10 tested samples (83.33%) a multifocal infiltrate was observed in the liver parenchyma. Hepatocyte damage ranged from mild intracellular edema and vacuolar degeneration to hepatocyte necrosis. A similar finding was established in people with hepatitis E (Zhao *et al.* 2001).

The dominant cell population in the mononuclear cellular infiltrate were CD3 positive lymphocytes. The population of CD79α positive lymphocytes was less frequent. Liver injury can be attributed to immune-mediated damage by cytotoxic T cells which are within the CD3+ cell subpopulation. Similar findings by Agrawal and colleagues and Prabhu and colleagues (Agrawal *et al.* 2012, Prabhu *et al.* 2010) were proven in cases of hepatitis E in humans.

Generally, damage to liver cells by viruses may occur as a result of the direct effects of the virus or indirectly as a result of the antiviral immune response of the host, or a combination of both. It is possible that the powerful immune response leads to the efficient removal of viruses and liver damage of various degree, a weaker immune response leads to the persistence of the virus and liver damage of lower intensity (Srivastava *et al.* 2007). The mechanisms that regulate the intensity of the immune response are probably crucial in defending the body from the virus.

Although many studies have described histological alterations of the swine liver, little is known about the mechanism of cell damage or the type of local immune response by the host cells to swine HEV. Demonstration of the immunophenotype of infiltrating lymphocytes in swine liver tissue infected with HEV, as performed in this study, has not been reported previously.

The observed presence of apoptotic bodies in liver of infected pigs was considered as a morphological manifestation of apoptosis. Apoptosis is established in experimental infections of pigs with porcine and human strains of hepatitis E virus (Halbur *et al.* 2001). It is known that apoptosis is also a physiologically active process in the regulation of the population of different cells, which is characterized by specific biochemical and morphological changes. Various signals within a cell or signals outside the cell can activate biochemical reactions in the cell which can result in apoptosis. In this complex process, cysteine proteinases are included. These are enzymes of the family of caspases, which play a key role in the process of apoptosis (Salvesen and Dixit 1997, Roulston *et al.* 1999). Massive necrosis of hepatocytes in the centrilobular and midzonal area was accompanied by a dense mononuclear infiltrate. It can be assumed that the number of lymphocytes is in direct relationship with the resultant necrotic process. This finding suggests that hepatocyte damage occurs as a result of immunoreactivity and highlights the importance of present lymphocytes in the pathogenesis of damage (Lee *et al.* 2007, Halbur *et al.* 2001, de Deus *et al.* 2008, Lee *et al.* 2008, de Deus *et al.* 2007, Meng *et al.* 1997). Studies on hepatitis E in humans have shown that immune-mediated liver damage is likely to arise as a result of the action of cytotoxic T lymphocytes and natural killer cells (NK cells) (Prabhu *et al.* 2011). The presence of activated Kupffer cells is due to increased synthesis of proinflammatory cytokines. An increased number of Kupffer cells was found in the liver of people with hepatitis E (Zhao *et al.* 2001).

The presence of TGF-β1 positive cells located in the mononuclear cell infiltrate in the liver parenchyma can be associated with apoptosis of hepatocytes, which was present in a third of tested pigs, and a reduced inflammatory response. This hypothesis is based on the fact that TGF-β1 induced apoptosis in the liver and bile ducts in a number of liver pathologies (Takiya *et al.* 1995, Quaresma *et al.* 2006).

Upon examination of pathological changes occurring in the liver of humans suffering from yellow fever, researchers revealed CD4+ T cells as the dominant cell population present in the mononuclear cell infiltrate, as well as hepatocytes which express TGF-β. It is believed that TGF-β is responsible for apoptosis of hepatocytes which in this disease was the dominant finding, as well as a weaker inflammatory response (Quaresma *et al.* 2006). A source of TGF-β1 in the liver are Kupffer cells, endothelial cells, hepatocytes, epithelial cells of the biliary duct and perisinusoidal stellate cells (Hinz *et al.* 2007). When hepatocytes undergo apoptosis they actively secrete cytokines and chemokines that activate the transmission signal to adjacent cells and release inflammatory cytokines and chemokines including TGF-β1. In our study, portal liver fibrosis was confirmed in two cases. According to some authors the release of TGF-β1 is the key event in the pathogenesis of fibrosis (Takiya *et al.* 1995, Kisseleva and Brenner 2008). TGF-β1 has multiple functions during organogenesis, tissue damage, and recovery. In the development of liver fibrosis not only increased synthesis of TGF-β1 is present, but also activation of latent to biologically active form is more intense (Friedman *et al.* 1999).

Conclusions

Our finding provides further evidence for an immune mediated pathogenesis in swine naturally infected with hepatitis E virus. Detected liver lesions, as well as the local immunological response, are similar to those detected in human HEV infection. Both morphological features, i.e. the dominant CD3+ T cell population in the mononuclear infiltrate, as well as the finding of apoptotic bodies in the liver of pigs naturally infected with HEV have not been reported previously.

Acknowledgements

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References

Agrawal V., Goel A., Rawat A., Naik S. & Aggarwal R. 2012. Histological and immunohistochemical features in fatal acute fulminant hepatitis E. *Indian J Pathol Microbiol*, **55**, 22-27.

Aprea G., Amoroso M.G., Di Bartolo I., D'Alessio N., Di Sabatino D., Boni A., Cioffi B., D'Angelantonio D., Scattolini S., De Sabato L., Cotturone G., Pomilio F., Migliorati G., Laliero G. & Fusco G. 2018. Molecular detection and phylogenetic analysis of hepatitis E virus strains circulating in wild boars in south-central Italy. *Transbound Emerg Dis*, **65** (1), e25-e31.

Banks M., Bendall R., Grierson S., Heath G., Mitchell J. & Dalton H. 2004. Human and porcine hepatitis E virus strains, United Kingdom. *Emerg Infect Dis*, **10** (5), 953-955.

Bansal M., Kaur S., Deka D., Singh R. & Gill J.P.S. 2017. Seroepidemiology and molecular characterization of hepatitis E virus infection in swine and occupationally exposed workers in Punjab, India. *Zoonoses Public Health*, **64** (8), 662-672.

Bouwknegt M., Rutjes S.A., Reusken C., Stockhofe-Zurwieden N., Frankena K., de Jong M.C., de Roda Husman A.M. & Poel W.H. 2009. The course of hepatitis E virus infection in pigs after contact-infection and intravenous inoculation. *BMC Vet Res*, **5**, 7.

Caruso C., Peletto S., Rosamilia A., Modesto P., Chiavacci L., Sona B., Balsamelli F., Ghisetti V., Acutis P.L., Pezzoni G., Brocchi E., Vitale N., Masoero L. 2017. Hepatitis E virus: a cross sectional serological and virological study in pigs and humans at zoonotic risk wthin a high density pig farming area. *Transbound Emerg Dis*, **64** (5), 1443-1453.

Cowan S.T. & Steel K.T. 1993. Manual for identification of medical bacteria. Cambridge University Press, Bentley House, London.

Cruickshank R., Duguid J.P., Marmion B.P. & Swain R.H.A. 1975. Medical microbiology. 12th ed. Churchill Livingstone, Edinburgh, London.

de Deus N., Peralta B., Pina S., Allepuz A., Mateu E., Vidal D., Ruiz-Fons F., Martin M., Gortazar C. & Segales J. 2008. Epidemiological study of hepatitis E virus infection in European wild boars (*Sus scrofa*) in Spain. *Vet Microbiol*, **129** (1-2), 163-170.

de Deus N., Seminati C., Pina S., Mateu E., Martin M., Segales J. 2007. Detection of hepatitis E virus in liver, mesenteric lymph node, serum, bile and faeces of naturally infected pigs affected by different pathological conditions. *Vet Microbiol*, **119** (2-4), 105-114.

Di Bartolo I., Martelli F., Inglese N., Pourshaban M., Caprioli A., Ostanello F. & Ruggeri F.M. 2008. Widespread diffusion of genotype 3 hepatitis E virus among farming swine in Northern Italy. *Vet Microbiol*, **132** (1-2), 47-55.

Erker J.C., Desai S.M., Schlauder G.C., Dawson G.J. & Mushahwar I.K. 1999. A hepatitis E variant from the United States: molecular characterization and transmission in cynomolgus macaques. *J General Virol*, **80**, 681-690.

Forgach P., Nowotny N., Erdelyi K., Boncz A., Zentai J., Szucs G., Reuter G. & Bakonyi T., 2010. Detection of hepatitis E virus in samples of animal origin collected in Hungary. *Vet Microbiol*, **143** (2-4), 106-116.

Friedman S.L. 1999. Cytokines and fibrogenesis. *Semin Liver Dis*, **19** (2), 129-140.

Halbur P.G., Kasorndorkbua C., Gilbert C., Guenette D., Potters M.B., Purcell R.H., Emerson S.U., Toth M.E. & Meng X.J. 2001. Comparative pathogenesis of infection pigs with hepatitis E viruses recovered from a pig and a human. *J Clin Microbiol*, **39** (3), 918-923.

Hinz B., Phan S.H., Thannickal V.J., Galli A., Bochaton-Piallat M.L. & Gabbiani G. 2007. The myofibroblast – one function, multiple origins. *Am J Pathol*, **170** (6), 1807-1816.

Kekarainen T., Sibila M. & Segales J. 2006. Prevalence of swine Torque teno virus in post-weaning multisystemic 236 wasting syndrome (PMWS)-affected and non-PMWS-affected pigs in Spain. *J Gen Virol*, **87**, 833-837.

Kisseleva T. & Brenner D.A. 2008. Mechanisms of fibrogenesis. *Exp Biol Med (Maywood)*, **233** (2), 109-122. doi: 10.3181/0707-MR-190.

Lee S.H., Kang S.C., Kim D.Y., Bae J.H. & Kim J.H. 2007. Detection of swine hepatitis E virus in the porcine hepatic lesion in Jeju Island. *J Vet Sci*, **8** (1), 51-55.

Lee Y.H., Ha Y., Ahn K.K. & Chae C. 2008. Localisation of swine hepatitis E virus in experimentally infected pigs. *Vet J*, **179** (3), 417-421.

Lukač B., Knežević A., Milić N., Krnjaić D., Veljović Lj., Milićević V., Zorić A., Đurić S., Stanojević M. & Nišavić J. 2016. Molecular detection of PCV2 and PPV in pigs in Republic of Srpska, Bosnia and Herzegovina. *Acta Veterinaria-Beograd*, **66** (1), 51-60.

Meng X.J., Purcell R.H., Halbur P.G., Lehman J.R., Webb D.M., Tsarevam T.S., Haynes J.S., Thacker B.J. & Emerson S.U. 1997. A novel virus in swine is closely related to the human Hepatitis E virus. *Proc Natl Acad Sci U S A*, **94** (18), 9860-9865.

Meng X.J. 2003. Swine hepatitis E virus: cross species infection and risk in xenotransplantation. *Curr Top Microbiol Immunol*, **278**, 185-216. doi:10.1007/978-3-642-55541-1_7.

Okamoto H. 2007. Genetic variability an evolution of hepatitis E virus. *Virus Research*,**127** (2), 216-228.

Oleksiewicz M.B., Botner A., Madsen K.G. & Storgaard T. 1998. Sensitive detection and typing of porcine reproductive and respiratory syndrome virus by RT-PCR amplification of whole viral genes. *Vet Microbiol*, **64**, 7-22.

Prabhu S.B., Gupta P., Durgapal H., Rath S., Gupta S.D., Acharya S.K. & Panda S.K. 2011. Study of cellular immune response against hepatitis E virus (HEV). *J Viral Hepatitis*, **18** (8), 587-594.

Purcell R.H. 1996. Hepatitis E virus. *In* Fields virology (Fields B.N., Knipe D.M., Howley P.M. eds). 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa. USA, 2831-2843.

Quaresma J.A., Barros V.L., Pagliari C., Fernandes E.R., Guedes F., Takakura C.F., Andrade H.F. Jr., Vasconcelos P.F. & Duarte M.I. 2006. Revisiting the liver in human yellow fever: virus induced apoptosis in hepatocytes associated with TGF-β, TNF-α and NK cells activity. *Virology*, **345** (1), 22-30.

Roić B., Terzić S., Florijančić T., Prpić J., Ozimec S., Jemeršić L., Bošković I., Jungić A. & Keros T. 2018. Preliminary serological and molecular investigation of selected viral pathogens in croatian cervid species. *Acta Veterinaria-Beograd*, **68** (1), 65-79.

Roulston A., Marcellus R.C. & Branton P.E. 1999. Virus and apoptosis. *Annu Rev Microbiol*, **53**, 577-628.

Salvesen G.S. & Dixit M.V. 1997. Caspase: intracellular signaling by proteolysis. *Cell*, **91** (4), 443-446.

Srivastava R., Aggarwal R., Jameel S., Puri P., Gupta V.K., Ramesh V.S., Bhatia S. & Naik S. 2007. Cellular immune responses in acute hepatitis E virus infection to the viral open reading frame 2 protein. *Viral Immunol*, **20** (1), 56-65.

Takiya S., Tagaya T., Takahashi K., Kawashima H., Kamiya M., Fukuzawa Y., Kobayashi S., Fukatsu A., Katoh K. & Kakumu S. 1995. Role of transforming growth factor β1 on hepatic regeneration and apoptosis in liver disease. *J Clin Pathol*, **48** (12), 1093-1097.

Tei S., Kitajima N., Tukahashi K. & Mishiro S. 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. *Lancet*, **362** (9381), 371-373.

Vasickova P., Psikal I., Wide F., Smitalova R., Bendova J., Pavlik I. & Kralik P. 2009. Detection and genetic characterisation of hepatitis E virus in Czech pig production herds. *Res Vet Sci*, **87** (1), 143-148.

Walewska-Zielecka B., Madalinski K., Jablonska J., Godzik P., Cielecka-Kuszyk J. & Litwinska B. 2008. Composition of inflammatory infiltrate and its correlation with HBV/HCV antigen expression. *World J Gastroenterology*, **14** (25), 4040-4046.

Zhao J., Wang S., Zhang T., Liu P., Zhou G.D., Sun Y.L., & Yin T.Y. 2001. [Pathological and virological studies of the liver tissues from the patients with sporadic hepatitis E]. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi*, **15** (3),205-207.