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Immunohistochemical analysis of ORF2 protein and ORF3 protein of hepatitis E virus in livers of swine in Mato Grosso state, Brazil[1]

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ABSTRACT.- Campos C.G., Pavelegini L.A.D., Pereira A.H.B., Souza M.A. & Pescador C.A. 2022. **Immunohistochemical analysis of ORF2 protein and ORF3 protein of hepatitis E virus in livers of swine in Mato Grosso state, Brazil.** *Pesquisa Veterinária Brasileira 42:e07091, 2022*. Laboratório de Patologia Veterinária, Universidade Federal de Mato Grosso, Av. Fernando Corrêa da Costa 2367, Bairro Boa Esperança, Cuiabá, MT 78069-900, Brazil. E-mail: carolpescador@yahoo.com.br

Hepatitis E is an emerging zoonotic disease caused by hepatitis E virus (HEV). Immunohistochemistry (IHC) can be used to verify viral presence in human and swine livers. The aim of this study was to comparatively analyze the immunolabeling of the ORF2 protein (pORF2) versus the ORF3 protein (pORF3) of HEV in swine livers from subsistence farms in the state of Mato Grosso, Brazil. This study included 25 liver samples formalin fixed paraffin embedded tissue block from a published molecular detection and immunohistochemistry (IHC) study, which used the HEV pORF3 protein, demonstrating 4% (1/25) of positive immunolabeling and 96% (24/25) negative, in contrast to the molecular exam that showed 24% (6/25) of liver samples positive and 76% (19/25) negative. In order to increase the sensitivity of the IHC technique, these samples were analyzed using the antibody for the detection of HEV pORF2, showing 24% (6/25) immunolabeling positive and 76% (19/25) negative, equivalent to the result of molecular analysis on corresponding samples. Thus, the use of antibody to pORF2 increased the number of HEV cases detectable in the IHC by 600%. The IHC added to molecular techniques can be used as a tool for monitoring viral presence in swine livers, constituting a sensitive diagnostic methodology when liver samples fixed in formalin and embedded in paraffin are available.

INDEX TERMS: Immunohistochemistry, pORF2 HEV, protein, hepatitis E virus, liver, swine, emergent zoonosis, Brazil.

RESUMO.- [**Análise imuno-histoquímica da proteína ORF2 e proteína ORF3 do vírus da hepatite E em fígados de suínos no estado de Mato Grosso, Brasil.**] A hepatite E é uma enfermidade emergente de caráter zoonótico causada pelo Vírus da Hepatite E (HEV). A imuno-histoquímica (IHQ) pode ser utilizada para verificar a presença viral em fígados de humanos e suínos. O objetivo deste estudo foi analisar comparativamente a imunomarcação da proteína ORF2 (pORF2) versus proteína ORF3 (pORF3) de HEV em fígados de suínos de criatórios de subsistência no estado de Mato Grosso, Brasil. Este trabalho incluiu 25 amostras de fígados de suínos fixados em formol e embebidos em parafina provenientes de um estudo publicado de detecção molecular e imuno-histoquímica (IHQ), que utilizou pORF3 de HEV, demonstrando 4% (1/25) de imunomarcação positiva e 96% (24/25) negativa, em contraste com o exame molecular que apresentou 24% (6/25) das amostras de fígado positivas e 76% (19/25) negativas. Com o objetivo de aumentar a sensibilidade da técnica de IHQ, essas amostras foram analisadas utilizando o anticorpo para detecção da pORF2 de HEV, apresentando 24% (6/25) de imunomarcação positiva e 76% (19/25) negativa, equivalente ao resultado da análise molecular em amostras correspondentes. Desta forma, o uso do anticorpo para pORF2 ampliou o número de casos de HEV detectáveis na IHQ em 600%. A IHQ somada a técnica molecular pode ser utilizada como ferramenta

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de monitoramento da presença viral em fígados de suínos, constituindo uma metodologia diagnóstica sensível quando há disponibilidade de amostras de fígado fixadas em formol e embebidas em parafina.

TERMOS DE INDEXAÇÃO: Imuno-histoquímica, pORF2 HEV, proteína, hepatite E, fígado, suínos, zoonose emergente, Brasil.

INTRODUCTION

Hepatitis E is an emerging disease caused by the hepatitis E virus (HEV) (Purdy et al. 2017). HEV belongs to the Hepeviridae family and infects mammals, birds, and fish. HEV variants in humans and swine belong to the genus *Orthohepevirus*, which is divided into four species (A-D) and eight genotypes (Wang & Meng 2021). The viral genome is formed by a single strand of RNA containing three discontinuous and partially overlapping open reading frames (ORFs). ORF1 encodes non-structural proteins, ORF2 encodes the viral capsid structural protein, and ORF3 encodes a phosphoprotein involved in virion morphogenesis and output. The presence of ORF4, identified in genotype 1 but not in others, has also been discussed (Montpellier et al. 2018, Kenney & Meng 2019).

HEV infections can be zoonotically transmitted and should be considered in public health (Wang & Meng 2021). An important route of transmission is the consumption of infected pork products that have been undercooked or consumed without cooking (Di cola et al. 2021). Pigs do not develop overt clinical signs and generally show only minimal to moderate evidence of liver inflammation (Cullen & Lemon 2019). In humans, HEV infection can also cause asymptomatic cases or acute hepatitis with severe and disseminated hepatocellular necrosis (Cullen & Lemon 2019). HEV infection can also extrahepatic manifestations, including neurological disorders and kidney injury (Primadharsini et al. 2021). Patients with liver disease and pregnant women are may be more vulnerable to HEV infection (Wang & Meng 2021).

Detection of HEV using immunohistochemistry is related to the production of viral proteins in certain cell types (Lee et al. 2009). Previous IHC studies have been described viral antigen detection in paraffin-embedded human HEV infected livers (Gupta et al. 2012, Friedman et al. 2016, Lenggenhager et al. 2017) and swine (Ha & Chae 2004, Lee et al. 2009, Lana et al. 2014, De Souza et al. 2018).

Lenggenhager et al. (2017) carried out a comparative immunohistochemical study to identify and localize viral proteins in human livers using different polyclonal and monoclonal antibodies to the three ORFs of the viral genome, relating the sensitivity of the immunohistochemical technique to molecular detection, *in situ* hybridization, and immunofluorescence techniques to establish reliable diagnoses of HEV infection. However, immunohistochemical studies comparing the differences in immunolabeling intensity between the use of HEV ORF2 and ORF3 proteins in swine livers have not yet been reported in the literature.

The aim of this study was to comparatively analyze the immunolabeling of ORF2 protein (pORF2) versus ORF3 protein (pORF3) from hepatitis E virus in swine livers from subsistence farms in the state of Mato Grosso, Brazil.

MATERIALS AND METHODS

Sampling. In this study, immunohistochemistry (IHC) was performed using the HEV ORF2 protein. For this, 25 paraffin swine (*Sus scrofa domesticus*) liver blocks from a previous study of HEV (Lana et al. 2014) were selected.

Immunohistochemistry. A monoclonal anti-HEV ORF2 Clone 1EC (Millipore Corporation) was used as the primary antibody. For standardization of the positive control of the reaction, a histological section of the swine liver which was positive for HEV RNA in the molecular examination was used. For negative control the primary antibody was replaced by phosphate-buffered saline pH 7.4 (PBS) in each case. In addition, a histological section of the healthy liver bovine was used.

Data on the dilution of antibodies, antigen retrieval, detection method, and chromogen for the detection of pORF2 of HEV are summarized in Table 1.

Qualitative analysis. The liver sections were evaluated using optical microscopy (Axio Imager A2, Carl Zeiss) to verify the presence, intensity, and location of immunolabeling of the HEV antigen according to the zones of the liver lobes and the cell types involved. The positivity criteria established in this study were based on morphological characteristics and defined by immunolabeling in hepatocytes (cytoplasmic and/or nuclear), with a granular appearance and intensely brownish color (Lenggenhager et al. 2017, De Souza et al. 2018).

RESULTS

In this study, IHC results with monoclonal primary antibody to HEV pORF2 in 25 liver samples showed 24% (6/25) positive and 76% (19/25) negative immunolabeling. The samples positive in the immunohistochemical analysis in this study were the same samples positive in the molecular analysis performed by Lana et al. (2014).

The characteristics of the immunolabeling pattern observed in this study included a distinguishable and specific granular aspect, with an intense brownish coloration located in the cytoplasm of intact hepatocytes (Fig.1). Hepatocytes with immunolabeled cytoplasm showed a multifocal and random distribution, often in a pattern of linear bands extending through the liver lobes or sometimes focally grouped (Fig.2) without background staining. Immunolabeling was restricted to the cytoplasm of hepatocytes, and immunolabeling with a nuclear or reticular location was not observed in any of the samples. None of the negative control sections showed evidence of immunolabeling.

Table 1. Immunohistochemical protocols for the detection of pORF2 of HEV

Antibody	Clone/Code	Antigen retrivel	Dilution	Detection methods	Chromogen
HEV ORF2	1EC (Millipore Corporation)	20 min/100oC Tris-EDTA buffer pH 9.0	1:500	MACH4 HRP Polymer (Biocare)	DAB (Dako)

pORF2 = ORF2 protein, HEV = hepatitis E virus, MACH4 universal HRP polymer = streptavidin biotin horseradish peroxidase (Biocare), DAB = 3,3′ diaminobenzidine (Dako).

DISCUSSION

The present study compared the immunohistochemical findings of hepatitis E in pigs using anti-HEV pORF 2 and ORF3 antibodies in previously fixed and paraffin-embedded liver samples. Studies of immunohistochemical detection of HEV in swine are still restricted in the literature, showing variability among the findings and lack of standardization among the antibodies used. With the constant advances in immunohistochemical techniques for HEV detection described in the human literature and the possibility of differences between the immunolabeling intensity according to the genomic region represented by the chosen antibody (Lenggenhager et al. 2017), we formulated the question of that these differences could also be representative in relation to the swine species. The isolated use of commercial antibodies for immunolabeling of HEV ORF2 protein in swine livers has been previously reported by Ha & Chae (2004) and Lee et al. (2009). However, there are no reports of immunohistochemical studies comparing the use of different antibodies to detect pORF2 and pORF3 in corresponding samples from swine livers.

To assess the characteristics of HEV immunolabeling in human livers, a comparative study carried out by Lenggenhager et al. (2017) showed that the immunolabeling intensity of anti-pORF2 antibodies was higher than that of pORF3 and pORF1 antibodies. A comparative study carried out by Gupta et al. (2012) also reported an intense positive reaction and a greater proportion of human hepatocytes using antibodies to detect the pORF2 than pORF3. The ORF2 is located at the 3' end of the viral genome and encodes the major viral capsid protein. It has been shown that this protein also contains immunogenic epitopes that induce cell-mediated immunity (Wang & Meng 2021).

These findings described in humans corroborate the results observed in this study, since the immunolabeling analysis comparing the results of different primary antibodies confirmed that the use of pORF2 increased the number of cases of HEV infection detectable by immunohistochemistry, with the corresponding difference being 600% favorable to ORF2 in relation to ORF3 in the analyzed samples**.** Despite a sample of only 25 pigs, the immunohistochemical findings with the use of the pORF2 were the same to the molecular findings by Lana et al. (2014) in the corresponding sample of the livers.

According to Purcell (1996), differences between genomic regions can be correlated with the amount of viral capsid proteins (pORF2) being expressed in host cells during infection, since the epitopes encoded by the ORF2 region are more conserved (90.5%) than the epitopes contained in ORF3 (73.5%). Ankavay et al. (2019) reported that ORF2-derived proteins are extremely stable in infected humans and may represent markers of the evolution of HEV infection. Thus, the use of an pORF2 antibody increased the sensitivity of virus detection in porcine livers constituting a recommended diagnostic methodology for retrospective studies evaluating viral circulation when liver samples fixed in formalin and embedded in paraffin are available.

The epidemiological design of Lana et al. (2014) was based on a case-control study that aimed to compare commercial and subsistence farms (family scale) and used pigs aged three to four months. The selection of this age group was intentionally limited and is related to the dynamics of HEV infection via the fecal-oral route in swine. Although the percentage of infected animals varies according to age, the virus can be identified in animals aged one to five months with a higher prevalence in pigs between three and four months of age, due to the decrease in maternal antibodies and the increased probability of infection through fecal contamination of the environment, food and water (Williams et al. 2001, De Deus et al. 2008). Once swine become infected, the virus reaches the liver through the portal vein, replicating in the cytoplasm of hepatocytes (Choi & Chae 2003), and can be detected through molecular analysis or immunohistochemistry during this period.

Immunohistochemistry can provide important information about the spatial location of the virus during infection, helping to understand the pathogenesis (Lenggenhager et al. 2017). However, to characterize the spatial localization of HEV proteins

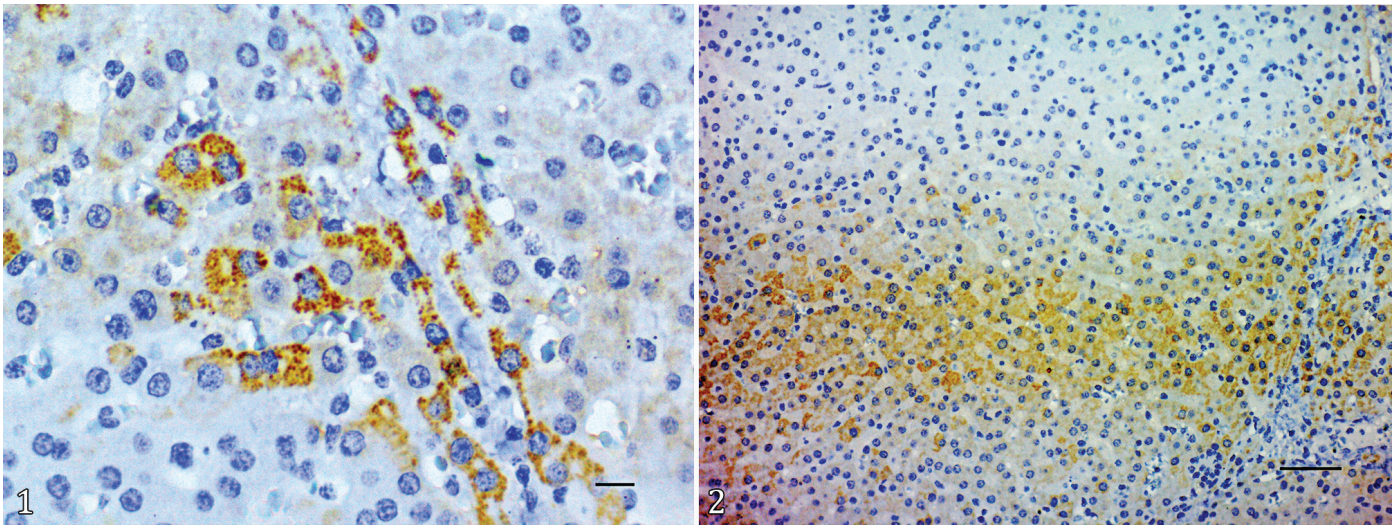

Fig.1-2. (**1**) Swine, liver. Detection of hepatitis E virus (HEV) antigens ORF2 in formalin-fixed, paraffin embedded. The hepatocytes with cytoplasmic immunostaining were distributed at random in the liver. IHC, obj.63x, bar = 10µm. (**2**) Swine, liver. The immunostained hepatocytes showed a brown cytoplasmic staining of hepatitis E virus (HEV) with a linear distribution pattern. IHC, obj.20x, bar = 50µm.

is important to consider the large genetic variability viral and the wide range of domestic and wild hosts that can be infected, determining different patterns of histopathological lesions in the liver (Cullen & Lemon 2019), and differences in the spatial location of immunolabeling in hepatocytes between species (Ha & Chae 2004, Lenggenhager et al. 2017).

Immunohistochemical studies using only isolated commercial antibodies to HEV ORF2 protein were previously reported in swine, revealing of pORF2 immunostaining with spatial localization in hepatocyte cytoplasm (Ha & Chae 2004, Lee et al. 2009), with minimal or no histopathological lesions, presenting characteristics and spatial location similar to those observed in this study. In a study by Lana et al. (2014), no statistical association was observed between the presence of viral RNA in the liver and the occurrence of microscopic lesions (mild lymphoplasmacytic periportal hepatitis), which reiterates the immunolabeling observed in intact hepatocytes in this study; however, in contrast, De Souza et al. (2018) reported the occurrence of marked histological lesions in swine hepatocytes associated with cytoplasmic immunolabeling of HEV using the primary antibody to pORF3. In humans are reported severe and disseminated hepatocellular necrosis associated with an expression and subcellular localization of pORF2 in human hepatocytes with cytoplasmic localization and in the nucleus of hepatocytes (Friedman et al. 2016, Lenggenhager et al. 2017, Ankavay et al. 2019). According to Ankavay et al. (2019), the infectious form of pORF2 can be translocated to the nucleus of the infected cell to control cell functions, promote viral replication, or alter the antiviral response of the infected cell. There are no reports of HEV immunostaining in porcine hepatocyte nuclei. We assume that these differences in the spatial location of viral proteins in hepatocytes may be associated with the occurrence of mild to moderate (swine) and severe (human) histopathological lesions.

CONCLUSION

This study demonstrated that the monoclonal primary antibody to ORF2 protein (pORF2) of hepatitis E virus (HEV) increased the sensitivity of virus detection in liver samples from pigs when compared to the polyclonal primary antibody to pORF3, providing results equivalent to molecular findings in corresponding samples.

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REFERENCES

Ankavay M., Montpellier C., Sayed I.M., Saliou J.-M., Wychowski C., Saas L., Duvet S., Aliouat-Denis C.-M., Farhat R., Masson d'Autume V., Meuleman P., Dubuisson J. & Cocquerel L. 2019. New insights into the ORF2 capsid protein, a key player of the hepatitis E virus lifecycle. Scient. Rep. 9(1):1-15. <https://dx.doi.org/10.1038/s41598-019-42737-2>

Choi C. & Chae C. 2003. Localization of swine hepatitis E virus in liver and extrahepatic tissues from naturally infected pigs by in situ hybridization. J. Hepatol. 38(6):827-832. <https://dx.doi.org/10.1016/S0168-8278(03)00115-6> <PMid:12763377>

Cullen J.M. & Lemon S.M. 2019. Comparative pathology of hepatitis A virus and hepatitis E virus infection. Cold Spring Harb. Perspect. Med. 9(4):a033456. <https://dx.doi.org/10.1101/cshperspect.a033456> <PMid:29712683>

De Deus N., Casas M., Peralta B., Nofrarías M., Pina S., Martín M. & Segalés J. 2008. Hepatitis E virus infection dynamics and organic distribution in naturally infected pigs in a farrow-to-finish farm. Vet. Microbiol. 132(1/2):19-28. <https://dx.doi.org/10.1016/j.vetmic.2008.04.036> <PMid:18562132>

De Souza A.J.S., Malheiros A.P., Soares M.C.P., Gomes-Gouvêa M.S., Pinho J.R.R., Pereira W.L.A. & Sá L.R.M. 2018. Hallmarks of liver lesions in pigs naturally infected by hepatitis E virus genotype 3. Pesq. Vet. Bras. 38(1):65-70. <https://dx.doi.org/10.1590/1678-5150-pvb-5368>

Di Cola G., Fantilli A.C., Pisano M.B. & Ré V.E. 2021. Foodborne transmission of hepatitis A and hepatitis E viruses: A literature review. Int. J. Food Microbiol. 338(1):108986. <https://dx.doi.org/10.1016/j.ijfoodmicro.2020.108986> <PMid:33257099>

Friedman L.S., Lee S.R., Nelson S.B. & Masia R. 2016. Case 36-2016. A 50-Year-Old Man with Acute Liver Injury. N. Engl. J. Med. 375(21):2082-2092. <https://dx.doi.org/10.1056/nejmcpc1610099> <PMid:27959737>

Gupta P., Jagya N., Pabhu S.B., Durgapal H., Acharya S.K. & Panda S.K. 2012. Immunohistochemistry for the diagnosis of hepatitis E virus infection. J. Viral Hepat. 19(2):e177-e183. <https://dx.doi.org/10.1111/j.1365-2893.2011.01498.x> <PMid:22239516>

Ha S.-K. & Chae C. 2004. Immunohistochemistry for the detection of swine hepatitis E virus in the liver. J. Viral Hepat. 11(3):263-267. <https://dx.doi.org/10.1111/j.1365-2893.2004.00498.x> <PMid:15117329>

Kenney S.P. & Meng X.-J. 2019. Hepatitis E virus genome structure and replication strategy. Cold Spring Harb. Perspect. Med. 9(1):a031724. <https://dx.doi.org/10.1101/cshperspect.a031724> <PMid:29530948>

Lana M.V.C., Gardinali N.R., Cruz R.A.S., Lopes L.L., Silva G.S., Caramori Júnior J.G., Oliveira A.C.S., Souza M.A., Colodel E.M., Alfieri A.A. & Pescador C.A. 2014. Evaluation of hepatitis E virus infection between different production systems of pigs in Brazil. Trop. Anim. Health Prod. 46(2):399-404. <https://dx.doi.org/10.1007/s11250-013-0503-3> <PMid:24272392>

Lee Y.-H., Ha Y., Ahn K.-K., Cho K.-D., Lee B.H., Kim S.-H. & Chae C. 2009. Comparison of a new synthetic, peptide-derived, polyclonal antibody-based, immunohistochemical test with in situ hybridisation for the detection of swine hepatitis E virus in formalin-fixed, paraffin-embedded tissues. Vet. J. 182(1):131-135. <https://dx.doi.org/10.1016/j.tvjl.2008.05.009> <PMid:18701328>

Lenggenhager D., Gouttenoire J., Malehmir M., Bawohl M., Honcharova-Biletska H., Kreutzer S., Semela D., Neuweiler J., Hürlimann S., Aepli P., Fraga M., Sahli R., Terracciano L., Rubbia-Brandt L., Müllhaupt B., Sempoux C., Moradpour D. & Weber A. 2017. Visualization of hepatitis E virus RNA and proteins in the human liver. J. Hepatol. 67(3):471-479. <https://dx.doi.org/10.1016/j.jhep.2017.04.002> <PMid:28412294>

Montpellier C., Wychowski C., Sayed I.M., Meunier J.-C., Saliou J.-M., Ankavay M., Bull A., Pillez A., Abravanel F., Helle F., Brochot E., Drobecq H., Farhat R., Aliouat-Denis C.-M., Haddad J.G., Izopet J., Meuleman P., Goffard A., Dubuisson J. & Cocquerel L. 2018. Hepatitis E virus lifecycle and identification of 3 forms of the ORF2 capsid protein. Gastroenterology 154(1):211-223.e8. <https://dx.doi.org/10.1053/j.gastro.2017.09.020> <PMid:28958858>

Primadharsini P.P., Nagashima S. & Okamoto H. 2021. Mechanism of cross-species transmission, adaptive evolution and pathogenesis of hepatitis E virus. Viruses 13(5):909. <https://dx.doi.org/10.3390/v13050909> <PMid:34069006>

Purcell R.H. 1996. Hepatitis E virus, p.2831-2843. In: Fields B.N., Knipe D.M. & Howley P.M. (Eds), Fields Virology. 3rd ed. Lippincott-Raven, Philadelphia.

Purdy M.A., Harrison T.J., Jameel S., Meng X.-J., Okamoto H., Van der Poel W.H.M. & Smith D.B. 2017. ICTV virus taxonomy profile: Hepeviridae. J. Gen. Virol. 98(11):2645-2646. <https://dx.doi.org/10.1099/jgv.0.000940> <PMid:29022866>

Wang B. & Meng X.-J. 2021. Hepatitis E virus: host tropism and zoonotic infection. Curr. Opin. Microbiol. 59:8-15. <https://dx.doi.org/10.1016/j.mib.2020.07.004> <PMid:32810801>

Williams T.P., Kasorndorkbua C., Halbur P.G., Haqshenas G., Guenette D.K., Toth T.E. & Meng X.J. 2001. Evidence of extrahepatic sites of replication of the hepatitis E virus in a swine model. J. Clin. Microbiol. 39(9):3040-3046. <https://dx.doi.org/10.1128/JCM.39.9.3040-3046.2001> <PMid:11526125>