Theses of doctoral (PhD) dissertation

Detection of atypical porcine pestivirus, mapping of its prevalence and genetic characterization in Hungary

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1. Background and objectives of the doctoral thesis

Atypical porcine pestivirus (APPV), classified in the genus *Pestivirus*, was first identified in the USA in 2015. Since then, it has been proven that the virus has spread worldwide in both domestic pigs and wild boar herds. APPV was proved to be the causative agent of A-II type congenital tremor (CT) which is a long known disease of pigs in Hungary. Before our investigation, there were no evidence of the presence of APPV among Hungarian pig herds.

During our research of APPV, which is the basis of the thesis, we set ourselves the goal of gathering information about the presence and prevalence of a virus that had not been identified in Hungary before our research, and to find out what kind of connection it has with the occurrence of congenital tremor in newborn piglets, which has been observed in our country for a long time. Our goal was the

(i) detection of APPV in samples from various pig farms in Hungary,

(ii) partial genome sequencing and phylogenetic analysis of the collected APPV strains, which can help in understanding their origin, spread and genome evolution,

(iii) carrying out targeted, national prevalence studies to assess the spread of infection,

(iv) identification of the involvement of individual age groups in the infected herds, the rate of infection, and the dynamics of virus spread within the herd,

(v) application of an RNA-based *in situ* hybridization method (RNAscope) for the detection of the virus in tissue lesions, with particular regard to the identification of the affected cell types in the testicular tissue and the brain.

2. Summary

Pestiviruses mostly infect ruminants and swine and before had four recognized species: the viruses that cause viral diarrhea in cattle (bovine viral diarrhea virus, BVDV-1 and BVDV-2), the border disease virus that infects sheep and goats (border disease virus, BDV) and a significant herd pathogen, the classical swine fever virus (CSFV), which last occurred in wild boar in Hungary in 2009, and the restrictive measures were lifted in the affected areas in 2013. In the last decade, several new pestiviruses have been identified in various animal species, one of these being the atypical porcine pestivirus (APPV). Since the first identification of APPV, it has been demonstrated that the virus has spread worldwide in both domestic pigs and wild boar herds. Similar to classical swine fever, it can cause congenital tremor (CT) in newborn piglets when infected in utero. Unlike CSFV which is responsible for type A-I CT, APPV-infected piglets born with type A-II CT usually become clinically healthy by weaning age. Presumably, the reason for this phenomenon is the difference in the viral localization in the cerebellum, as APPV infects only the inner granular and molecular layer (including the Purkinje cells), so the neurons that die as a result of infection can be replaced by the cells of the intact, outer granular layer. The virus can be excreted for months in the faeces of already asymptomatic animals, as well as in the sperm of sexually mature males.

In 2016, we were the first to identify the virus in tissue samples from CT-affected pigs from domestic pig farms. We also identified APPV in archived tissue samples of CT-affected pigs from 2005, 2007 and 2010, which states that the virus has been present in domestic pig herds for at least 18 years. Between 2018 and 2022, we collected serum, processing fluid, and oral fluid samples from 26 farms in Hungary and one in Slovakia, which were screened using an APPV-specific RT-qPCR method. We detected the virus in a total of 18 farms. In the case of serum samples, we collected an average of 100 serum samples per farm from 2, 4, 6, 8, 10, 12, 14, and 18-week-old animals, as well as from gilts, sows of 2-and 4 parities, which were examined in pools of 5. Oral fluid samples were collected from 10- and 20-week-old animals. During cross-sectional studies with a large number of samples, we established a Hungarian prevalence of 66.67%, therefor two-thirds of the examined Hungarian pig farms were infected with the virus. The virus was detected in 6.3–50% of blood serum samples, 20–100% of testicular fluid samples and 10–100% of

oral fluid samples in the affected farms. Sows, 2- and 4-week-old piglets were all negative, 12-week-olds and gilts showed a low infection rate (4–6%), 6, 8, 14 and 18-week-olds (14–16%) and 10-year-olds (27%) showed the highest positivity rate in their serum samples. 41% of APPV-positive oral fluid samples came from 10-week-old and 59% from 20-week-old animals. In the case of two farms, APPV was only identified in the oral fluid samples, and in 5 infected farms, we could not detect the virus in processing fluid samples. Processing fluid samples proved to be reliable for diagnostic purposes when more than 5 samples from one farm were tested.

Based on our phylogenetic studies, the determined partial APPV sequences typically form a common phylogenetic group with European sequences, but some strains could be classified into a monophyletic group with a sample described in the Republic of Korea. Based on our results, the strains identified in Hungary do not form separate groups per farm, however, it happened that similar/identical strains were also identified in farms that were on average 100–300 km apart. Although we currently have no information about the trade relationship between these farms, it is possible that these strains reached the individual farms through trade within the country, possibly using gilts from a common source or even semen. Our results suggest that the main driving force behind the genetic diversity of APPV is the local, divergent evolution of strains, as well as the trade of infected, asymptomatic animals and/or reproductive material.

During the *in situ* hybridization examination of the testes of infected, 1–3-day-old pigs and a mature boar born with CT that later become asymptomatic, the interstitial Leydig cells, the peritubular myoid cells and the smooth muscle cells of the walls of medium-sized arteries were identified as the target cells of the virus. We also observed that, while in the case of young piglets, APPV could not be identified beyond the blood-testis (Sertoli) barrier, positive cells were also found in this area in the testicles of the mature boar, which, together with the infection of the bulbourethral gland and the prostate, explains the infected sperm discharge. We examined the presence of the virus in the cerebrum and cerebellum of these animals using an RNA *in situ* hybridization method. The genetic material of APPV was found in the inner granular and molecular layers of the cerebellum of piglets with CT, as well as in the Purkinje cells. In addition, we observed moderate staining bound to neurons of the cerebrum.

3. New scientific results

1. We were the first to identify atypical swine pestivirus in Hungarian and Slovakian herds from fresh and archived samples.

2. We were the first to carry out large-sample, representative, cross-sectional prevalence studies in domestic pig herds, and found that processing fluid samples, serum samples from 10-week-old pigs and oral fluid samples from 20-week-old animals are the most suitable for confirming the APPV infection of a herd.

3. For the first time, we determined the partial NS2–3 protein-coding region of the strains identified in Hungary and Slovakia, for comparative phylogenetic studies. We found that the domestic strains show great diversity, but they mostly form a phylogenetic group with viruses identified in Europe.

4. We were the first to develop an RNA-based *in situ* hybridization method (RNAscope) to investigate the localization of the virus in tissue.

5. We were the first to identify the involved cell types using RNAscope and IHC methods in the testicular tissue and accessory gonads of day-old piglets and a male with persistent APPV infection born with CT. We found that, in contrast to young animals, in sexually mature males the cells beyond the blood-testis barrier (Sertoli cells, germ cells) are also affected, the infection is not limited to the interstitial Leydig cells, the peritubular myoid cells around the seminiferous tubules and the smooth muscle cells located in the walls of medium-sized arteries.

4. Publications

- Dénes, L., Balka, G., 2022. Az atipikus sertés-pestivírus és az általa okozott reszketőkór Irodalmi összefoglaló. Magy. Állatorvosok Lapja 144, 591–602. doi:0.56385/magyallorv.2022.10.591-602, IF: 0,236
- Dénes, L., Ruedas-Torres, I., Szilasi, A., Balka, G., 2021. Detection and localization of atypical porcine pestivirus in the testicles of naturally infected, congenital tremor affected piglets. Transbound. Emerg. Dis. 1–9. doi:10.1111/tbed.14355, IF: 4,521
- Dénes, L., Biksi, I., Albert, M., Szeredi, L., Knapp, D.G., Szilasi, A., Bálint, Á., Balka, G., 2018. Detection and phylogenetic characterization of atypical porcine pestivirus strains in Hungary. Transbound. Emerg. Dis. 65, 2039–2042. doi:10.1111/tbed.12981, IF: 3,554