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Srebrno jezero, 30. maj – 01. jun 2019.

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FIRST DETECTION OF PORCINE EPIDEMIC DIARRHEA VIRUS IN MACEDONIA

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Abstract

In November, 2016, an outbreak of acute diarrhea in all pig categories was reported in one Macedonian farrow-to-finish pig farm. The present work describes the first clinical case and molecular detection of a porcine epidemic diarrhea virus (PEDV) in Macedonia.

We were informed by the farm veterinarians that the first clinical signs of profuse diarrhea were seen in fattening pigs with no response to the applied antibiotics. After one week, diarrhea swept across all age groups accompanied by increased mortality in lactating piglets. We visited the farm and clinical inspection of the animals was performed. During clinical examination in the farrowing rooms, diarrhea incidents were seen sporadically in 3-5% of litters having 1-2 diarrheic piglets. Three 5-10 days old piglets with profuse diarrhea were chosen, euthanized and necropsied. Gross lesions were limited to the small intestines and were suggestive for corona-virus infection. Fresh tissue samples from the piglet's small intestines were collected in order to be tested for PEDV and transmissible gastroenteritis virus (TGEV) by conventional multiplex reverse transcription polymerase chain reaction (RT-PCR). Results of the conventional multiplex RT-PCR showed that samples were positive for PEDV and negative for TGEV.

This is the first confirmed case of PEDV in Macedonia. However, more pig farms need to be tested to determine the real situation in the country. Further investigation regarding sequencing of the virus strain should be conducted in order to elucidate the possible origin of the PEDV outbreak in Macedonia.

Key words: Macedonia, pig farm, porcine epidemic diarrhea virus,

Introduction

Porcine epidemic diarrhea (PED) is a highly contagious and acute viral enteritic disease which affects pigs of all ages (Saif et al., 2012, Jung and Saif, 2015, Hanke et al., 2017). The causative agent is an enveloped single-stranded RNA virus that belongs to the genus *Alphacoronavirus* in the family *Coronaviridae* (Saif et al., 2012, Hanke et al., 2017). The PEDV causes acute diarrhea, vomiting, weight loss, and dehydration in all pig categories, while the most severe signs are seen in neonatal piglets in which mortality can reach up to 100%. (Carvajal et al., 2015). Viral replication occurrs in the cytoplasm of villous enterocytes throughout the small intestine causes villous atrophy and reduced enzymatic and absorptive capacity causing malabsorptive diarrhea (Saif et al., 2012). Although the rapid spread of a disease characterized by profuse watery diarrhoea affecting pigs of all ages allows the clinician to suspect that a viral agent is involved in the infection, differential diagnosis to identify PEDV at the laboratory is needed. Up to date the conventional or real-time PCR arė the most common diagnostic tests used for detection of PEDV in intestinal contents of the affected pigs (Song and Park, 2012).

The first clinical manifestation of PED in Europe was reported in 1971 in United Kingdom and later in 1978 in Belgium (Saif et al., 2012). Since the first description of the disease in Europe, within two decades it has been spread in several European countries causing sporadic and isolated outbreaks (Carvajal et al., 2015, Jung and Saif, 2015). Recent PED outbreaks in Europe have been reported in Portugal (Mesquita et al., 2015), Germany (Stadler et al., 2015), Italy (Bonioti et al., 2016), Austria (Steinrigl et al., 2015) Ukraine (Dastjerdi et al.. 2015), Belgium (Theuns et al., 2015), France (Grasland et al., 2014) and Serbia (Prodanov-Radulovic et al., 2017). In contrast to Europe, in the United states of America (USA) in April 2013, PEDV has spread throughout the country causing acute outbreaks of diarrhea with high mortality in suckling piglets (Chen et al., 2014). In Asia PEDV outbreaks with important losses in suckling pigs were first reported in 1982 and continued to occur in the 1990s and 2000s (Song et al., 2015). Phylogenetic analyses have shown that highly virulent strains involved in the outbreaks in USA in 2013 and in Asia revealed high nucleotid identity (Stadler et al., 2015). In 2014, a new PEDV strain OH851 associated with milder enteritis and lower mortality in suckling piglets in Ohio, USA was identified (Wang et al., 2014). This new variant includes some insertions and deletions in the S gene and is called S INDEL strain. Although, S INDEL and highly virulent non SINDEL strains are co-circulating in the USA, S INDEL are less frequently detected (Vlasova et al., 2013). The full genome sequencing of strains recovered from recent outbreaks in Germany (Stadler et al., 2015), France (Grasland et al., 2014), Slovenia (Toplak et al., 2015) and Belgium (Theuns et al., 2015) shows that they are all highly similar and closely related to S-INDEL strains found in USA.

Since the recent outbreak in Serbia (Prodanov-Radulovic et al., 2017), the PEDV has not been reported in the neighboring countries. In this study we report the first detection of PEDV in pigs on one Macedonian farrow-to-finish pig farm and provide information about clinical manifestation, gross pathology findings, molecular diagnosis and possible transmission route of PEDV.

Material and methods

An outbreak of diarrhea occurred in a farrow – to – finish pig farm at the end of November 2016. The farm had 700 sows and was located in the region of Vinica, Macedonia. The first clinical signs of acute profuse diarrhea were seen in high percentage (about 50 to 70%) of fattening pigs and with no response to the applied antibiotics. After one week of the first clinical cases in the fattening units, diarrhea was spread across all age groups followed by increased mortality in suckling pigs. Upon the farm manager request, we visited the pig farm in order to perform clinical examination. During clinical inspection we identified three 5-10 days old piglets with profuse diarrhea, euthanized and necropsied the piglets. Fresh tissue samples (small intestines) from the piglets were collected in order to be tested for PED and TGE viruses by PCR.

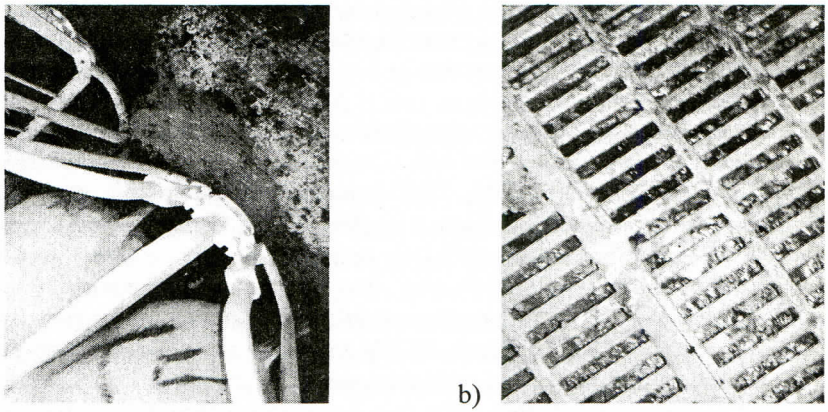
Small intestines of each piglet were dissected in a small pieces and together with intestinal contents were used for virus detection. About 30 mg of small intestines and intestinal content were homogenized in 700 µL PBS with Mixer Mill MM 200 (Retsch, Germany). After 5 minutes of centrifugation at 3000 rpm (2400 g), 200 µl of the clarified supernatant was used for the extraction of viral nucleic acids with Purelink™ Viral RNA/DNA Mini Kit (Invitrogen, USA) according to the manufacturer's instruction. Porcine epidemic diarrhea virus was detected by conventional multiplex RT-PCR using primers described by Song et al. (2006) that could detect PEDV and TGEV in pigs simultaneously. Amplification was done by the commercial OneStep RT-PCR kit (Qiagen, Germany). with primers that amplified the 651 bp fragment of spike protein (S) gene of PEDV P1 (TTCTGAGTCACGAACAGCCA, 1466–1485) and P2 (CATATGCAGCCTGCTCTGAA, 2097–2116) and 859 bp fragment for the S gene of TGEV (T1 (GTGGTTTTGGTYRTAAATGC, 16–35) and T2 (CACTAACCAACGTGGARCTA, 855–874). Composition of the reaction mix was as follows: 13.5 µl of nuclease free water, 5µl of 5 x PCR buffer, 1µl of dNTP mix (containing 10 mM of each dNTP), 1µl of each primer mix (with final concentration of 10µM of each primer), 1µl of one step RT-PCR enzyme mix and 2.5 µl of the extracted RNA. The thermal protocol included reverse transcription step at 500C for 30 min, followed by initial denaturation at 950C for 15 min, 40 cycles of denaturation at 940C for 30 seconds, annealing at 530C for 60 seconds, elongation at 720C for 60 seconds, and a final extension step at 720C for 10 minutes. The end point analysis of the RT-PCR products was performed on 1.5% agarose gel containing 1 µl ethidium bromide in 10 ml gel. Positive PCR controls for TGEV and PEDV were kindly provided by the Scientific Veterinary Institute Novi Sad, Serbia and originated from the field cases.

Results and discussion

Clinical findings on the investigated pig farm confirmed the presence of acute profuse diarrhea in all pig categories. On the day of farm visit diarrhea incidents in the fattening pigs were very low (1-2%). The highest incidents of watery greenish diarrhea accompanied by anorexia (about 50 to 70%) were found in sows in the gestation room (Figure 1). Diarrhea incidents were

very sporadic in the farrowing rooms (Figure 2 a, b) affecting 3-5% of the litters, while at weaning section profuse diarrhea was seen only in few pigs. Vomiting as a clinical sign was not found.

Figure 1. Diarrhea in gestation room



a) b)

Figure 2. a) Greenish diarrhea in gestating sows b) Yellowish diarrhea in farrowing

Gross pathology findings were identical in all 3 necropsied piglets. The lesions were suggestive of corona-virus infection and were limited to the digestive tract. Stomachs contained coagulated milk, and the small and large intestines were distended with thin transparent walls containing yellow watery fluid.

Conventional multiplex RT-PCR showed that all three gut samples were positive for PEDV and negative for TGEV (Figure 3).

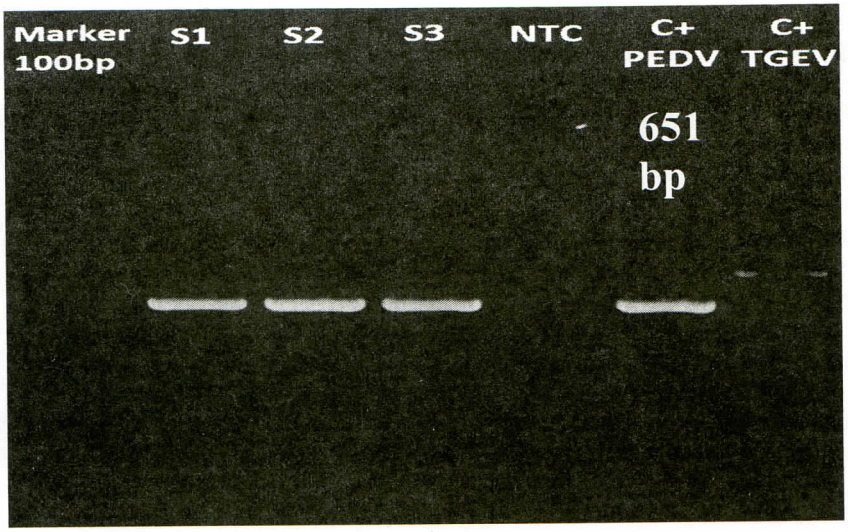


Figure 3. Conventional multiplex RT-PCR products of intestinal samples on 1.5% agarose gel. From left to right: Marker 100bp, positive PCR samples on PEDV S1, S2 and S3 at 651 bp. NTC - non template control, C+ PEDV - positive control and C+TGEV - positive control.

There are many enteritic diseases associated with severe losses in pig farms. The most commonly involved agents are *Escherichia coli*, *Clostridium perfrigens* type C, *Isospora suis* and enteritic viruses such as corona (PEDV, TGEV) and rotavirus. In this study clinical signs, macroscopic lesions and course of the disease in affected pig farm were indicative for coronavirus infection. The incubation period of PED is considered to range from1 to 8 days (Harris, 2012, Jung and Saif 2015, Song et al.,2015). The virus is replicated in the enterocytes of the small intestines and causes villous shortening, profuse diarrhea and dehydration in pigs (Carvajal et al., 2015). Other clinical signs associated with PEDV infection are vomiting, anorexia and fever where age of the pigs are closely related with the severity of the infection. Suckling piglets less then one week old are the most affected since they have slower turnover of the enterocytes (5-7 days) compared to three week-old piglets [2-3 days, (Song et al.,2015, Saif et al.,2012)]. According to the farm veterinarians, diarrhea firstly was detected in the fattening pigs and after 7-10 days rapidly spread to all pig categories. This finding is in accordance with recently reported PEDV outbreak in Serbia where the first clinical signs were seen in young fatteners (Prodanov-Radulovic et al., 2017). Similar data is reported during 2005-2006 PED outbreak in Italy within 50% of the fatteners pigs affected by watery diarrhea in the first days of the infection (Martelli et al.,2008). Similar outbreaks of PED were seen in pig farms in Germany (Stadler et al., 2015) and Austria (Steinrigl et al., 2015). The outbreak in Austria was characterized by mild clinical enteritis in fattening pigs which resolved in 2-3 days and no pigs have died from diarrhea or dehydration. In comparison to our findings, vomiting was observed in recent PED outbreaks in Serbia

(Prodanov-Radulovic et al., 2017), Italy (Martelli et al.,2008) and Germany (Stadler et al., 2015), but was not recorded in Austrian PEDV outbreak (Steinrigl et al., 2015). The mortality rate in suckling piglets in recent PEDV outbreaks in European countries was quite variable and ranged from 12 to 34% (Martelli et al.,2008, Stadler et al., 2015, Grasland et al., 2014). The manifestation of the disease and severity of the clinical signs in the herds are variable due to the level of the herd immune status, co-infections with other pathogens, management and biosecurity level (Stadler et al., 2015). All of these European isolates were found to be S INDEL strains similar with low virulent strains detected in USA (Chen et al., 2014). The only reported European PEDV non-INDEL strains of 99.8% genomic similarity with highly virulent US strains were recently recovered from the outbreaks in Ukraine (Dastjerdi et al.. 2015).

The possible route of PEDV entry is by fecal-oral contact with infected swine or by contaminated equipment, fomites or personnel. Dirty boots, clothes, hands or trucks can also spread the disease (Geiger et al., 2013). In the same period of an outbreak in the examined farm, four other pig farms reported the occurrence of acute diarrhea in the fattening pigs. One of these farms had imported breeding females from Austria ten days before clinical diarrhea occurred. These newly introduced animal might have already been PEDV infected and have shed the virus in the feces. Since all these five farms have used common slaughterhouse, our primary hypothesis was that contaminated and poorly cleaned and disinfected trucks for pig transportation from the slaughterhouse introduced the virus into the affected pig farm. Identically in recent outbreak in Serbia, transportation vehicles were considered to be a main source of PEDV infection (Prodanov-Radulovic et al., 2017). Moreover, in USA it was concluded that transport process of live pigs represented a source of PEDV transmission if appropriate hygiene measures are not carried out (Lowe et al., 2014).

After confirmation of PEDV by RT-PCR, managerial and biosecurity measures were taken. One of the main goal was to provide solid herd immunity by exposing all sows and gilts to the virus. Approximately three weeks of virus exposure is required for sows to develop sufficient amount of antibodies to protect their litters from the PEDV (Carvajal et al., 2015, Geiger and Connor, 2013). Piglets need to ingest sufficient amount of colostrums for the immunity to be protective. Neonatal piglet survival should begin 3 to 4 weeks after the process of virus exposure is begun. In our research aggressive feed-back with intestinal contents from clinically affected dead piglets at the age of 5-10 days was carried out in all pregnant sows and gilts. Feedback protocols include grounding the gut of affected piglets, dissolving in cold water and feeding the soup from one gut to 10-15 sows/gilts every day for 20 days or as many days as there is adequate material for sow/gilt exposure. For maximum viral content, infected piglets should be sacrificed within the first six hours of their clinical signs (Geiger and Connor, 2013). Feedback should be given until clinical signs are not observed or intestinal content from affected piglets is not available. In order to reduce viral load, sanitation and biosecurity are the best means of prevention (Geiger and Connor, 2013). In this context, the following steps should be included in terms of internal biosecurity at herd level: strict all in/all out movement of animals for farrowing and cleaned, disinfected and dried rooms between groups, cross fostering between litters should be minimize for several weeks, use of dry disinfectants which do not freeze for footbaths, washed

and disinfected equipment must be used between litters and rooms, scoring litters are processed last, hallways must be washed and disinfected with quick-lime spray and all rooms should be steamed and pressure washed to manure-free state then steamed again and then thoroughly disinfected. Regarding external biosecurity at herd level, rules such as quarantine, ban of entrance unwashed and not disinfected vehicles and strict visitor policies should be conducted without exception (Saif et al., 2012, Carvajal et al., 2015). However, there are no specific treatments for the control and potential eradication of the disease from the herd, preventive measures which preclude the introduction of the virus or new PEDV strains in the area, country or farm are of paramount importance (Carvajal et al., 2015).

According to the clinical aspects, gross lesion and laboratory findings this was the first confirmed case of PEDV in Macedonia. The transport vehicles were considered as a possible source of virus transmission. Nevertheless, this work was conducted in one pig farm and is not representative for whole country. More pig farms should be tested to determine the real situation of PEDV infection in the country. Additional research regarding genome sequencing of the virus strain should be performed in order to reveal the true origin of the outbreak.

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