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First detection and characterisation of porcine hemagglutinating encephalomyelitis virus in the Czech Republic

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Abstract: Porcine hemagglutinating encephalomyelitis virus (PHEV) is a highly neurovirulent coronavirus that invades the central nervous system in piglets. The incidence of PHEV among pigs in many countries is rising, and the economic losses to the pig industry may be significant. Serological studies suggest that PHEV is spread worldwide. However, no surveillance has been carried out in the Czech Republic. In this study, eight pig farms were screened for the presence of members of the *Coronaviridae* family with the use of reverse transcription PCR. A collection of 123 faecal samples and 151 nasal swabs from domestic pigs were analysed. In PHEV-positive samples, almost the complete coding sequence of the nucleocapsid gene was amplified and the acquired sequences were compared to those of geographically dispersed PHEV strains; phylogenetic analyses were also performed. PHEV was present in 7.9% of nasal swabs taken from different age categories of pigs. No other swine coronaviruses were detected. The amino acid sequence of the Czech PHEV strains showed 95.8–98.1% similarity to other PHEV reference strains in GenBank. PHEV strains collected from animals on the same farm were identical; however, strains from different farms have only exhibited only 96.7–98.7% amino acid sequence identity. Our study demonstrates the presence of PHEV in pigs in the Czech Republic. The Czech PHEV strains were evolutionarily closest to the Belgium strain VW572.

Keywords: PHEV; pig; RT-PCR; nucleocapsid; genetic analysis

Coronaviruses are enveloped viruses with single-stranded RNA genomes. Most members of the *Coronaviridae* family cause respiratory or gastrointestinal diseases in different mammalian species. Porcine hemagglutinating encephalomyelitis virus (PHEV) belongs to the *Betacoronavirus* genus together with murine hepatitis virus, bovine coronaviruses and also with dangerous human respiratory coronaviruses (SARS-CoV and MERS-CoV) (Masters 2006). The PHEV specifically infects swine and has a strong tropism for epithelial cells of the upper respiratory tract and for the central nervous system (CNS). The mode of transmission is through nasal secretions, and the virus spreads via peripheral nerves to the CNS. PHEV was first isolated and described as a causative agent of CNS disease (non-suppurative encephalomyelitis) in Canada in 1962 (Greig et al. 1962). The encephalomyelitis form of disease begins with vomiting which lasts for one to two days. However, it is not persistent and does not lead to dehydration. After one to three days hyperaesthesia and muscle shivers appear. Animals often move backwards; the sitting dog position is one of the typical symptoms. Later, animals are not able to stand; blindness, opisthotonus and nystagmus may be observed. The in-

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cubation period of CNS infection is four to seven days; morbidity and mortality in new-born piglets reaches 50–100% (Pensaert 2006).

Another clinical syndrome of PHEV infection is vomiting and wasting disease (VWD) in suckling piglets. The symptoms of VWD include repeating vomiting and nausea leading rapidly in new-born piglets to dehydration followed by cyanosis, later by coma and death. The mortality rate in infected piglets younger than three weeks can reach 100%; the surviving pigs stay stunted (Pensaert 2006).

Serological studies show that PHEV is spread worldwide; the number of seropositive animals ranges between 30–89% depending on the monitored region (Cartwright and Lucas 1970; Mengeling 1975; Hirano and Ono 1998). Infection is usually not clinically manifested, especially in older pigs. In herds with endemic occurrence of PHEV, the majority of piglets is protected by colostrum antibodies, which persist for approximately ten weeks (Pensaert et al. 1980).

However, epidemics of PHEV infection occur occasionally and their economic impact may be substantial. Just over a decade ago, a massive epidemic of encephalomyelitis with VWD symptoms occurred in Argentina with more than 3600 dead pigs (Quiroga et al. 2008). Neurological symptoms together with VWD in piglets were also detected in China (Gao et al. 2011). In the USA, a non-typical PHEV infection occurred in show pigs (six to eight months old) with influenza-like symptoms (Lorbach at al. 2017).

The objective of our study was to evaluate the occurrence of different coronaviruses in Czech pigs with a special emphasis on coronaviruses of unknown incidence. PHEV was the only coronavirus detected in samples of clinically healthy pigs on conventional Czech pig farms. Following the screening for the presence of coronaviruses, we confirmed the positive samples by sequencing. Phylogenetic analysis of the PHEV gene encoding the nucleocapsid protein was also carried out, and for the first time the evolutionary pattern of PHEV in the Czech Republic was characterised.

MATERIAL AND METHODS

Sample collection. Coronavirus detection was carried out on collection of 123 faecal samples (some of them pooled) and 151 nasal swabs from domestic pigs (*Sus scrofa* f. *domestica*) of different age categories. The samples were gathered on eight pig farms during 2016 and 2017. The faecal sample collection contained 44 samples from suckling piglets (of which 21 samples were collected from piglets with signs of acute diarrhoea), 18 samples from weaned four-to-ten-week-old piglets, 54 samples from 10-to-24-week-old fattening pigs and seven samples from saws. The nasal samples were taken with the use of nylon flocked swabs (Copan Diagnostics, USA) and transported in a tube containing phosphate-buffered saline (0.5 ml). The swabs were collected from 31 suckling piglets (of which six piglets showed signs of acute diarrhoea), 24 samples from weaned four-to-ten-week-old piglets (of which 10 animals suffered from vomiting), 90 samples from 10-to-24-week-old fattening pigs and six samples from saws.

Detection of the virus. Total RNA was extracted with the use of TRI Reagent (Sigma-Aldrich, USA) from the supernatant of a 10% suspension of faeces in phosphate-buffered saline or from nasal swabs according to the manufacturer's instructions. Reverse transcription was carried out using the ProtoScript II RT-PCR Kit with $d(T)_{23}VN$ primer mix (New England Biolabs, USA). In the subsequent PCR, Aptamer Hot Start Master Mix (TopBio, Czech Republic) was used. The first screening PCR for coronaviruses was performed with primers targeting the conserved region of the genome coding for RNA-dependent RNA polymerase (Tong et al. 2009). To increase the sensitivity of the reaction, the second round of PCR was carried out with a semi-nested pair of primers. In the case of a positive finding, the samples were subsequently analysed with primers specifically targeting expected members of the *Coronaviridae* family; namely, porcine epidemic diarrhea virus, PEDV; transmissible gastroenteritis virus, TGEV; porcine respiratory coronavirus, PRCV; porcine deltacoronavirus, PDCoV; and PHEV. The specificity of primers was verified on the specific virus strains (PEDV CAPM V-474, strain CV777; TGEV CAPM V-66, strain SH; PRCV V-355 CAPM, strain LUK; PHEV CAPM V-167, strain 67N) obtained from the Collection of Animal Pathogenic Microorganisms (CAPM, Veterinary Research Institute, Brno) which were also used as positive controls in the PCR. Amplified segments of the genome were sequenced to confirm the product specificity. Primers used for the screening of coronaviruses as well as sequencing primers are listed in Table 1.

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Table 1. Primers used for the screening and sequencing of coronavirus-positive samples

Target	Fw primer (5'-3')	Rev primer (5'-3')	Product (bp)	Reference
PanCor (screening)	ATGGGITGGGAYTATCCWAARTGTG	AATTATARCAIACAACISYRTCRTCA	460	Tong et al. 2009
PanCor – semi-nested (screening)	ATGGGITGGGAYTATCCWAARTGTG	CTAGTICCACCIGGYTTWANRTA	179	Tong et al. 2009
PEDV	TTGGCATTTCTACTACCTCGGA	AGATGAAAAGGTACTGCGTTCC	1327	Kubota et al. 1999
PEDV – nested	AGGAACGTGACCTCAAAGACATCCC	CCAGGATAAGCCGGTCTAACATTG	540	Kubota et al. 1999
TGEV (+PRCV)	GGGTAAGTTGCTCATTAGAAATAATGG	CTTCTTCAAAGCTAGGGACTG	1006 (PRCV ~350)	Kim et al. 2000
TGEV (+PRCV) – nested	TTGTGGTYTTGGTYGTAATKCC	GGCTGTTTGGTAACTAATTTRCCA	874 (PRCV ~220)	Kim et al. 2000
PDCoV	GTGGVTGTMTTAATGCACAGTC	TACTGYCTGTTRGTCATRGTG	440	Woo et al. 2012
PHEV (screening)	GTTTGGCCTCTTTWTCCTTWTG	TCAGAGCTAATAGATGGCACACC	322	Dong et al. 2014
PHEV (sequencing)	TAATACGTGGCCACCTCTACATCC	AGGTGCCGACATAAGGTTCATTCT	1632	Dong et al. 2014

PanCor = pan-coronavirus; PDCoV = porcine deltacoronavirus; PEDV = porcine epidemic diarrhea virus; PHEV = porcine hemagglutinating encephalomyelitis virus; PRCV = porcine respiratory coronavirus; TGEV = transmissible gastroenteritis virus

Sequence analyses. In PHEV-positive samples, acquired sequences were compared to those of geographically dispersed PHEV strains and sequences of other related coronaviruses deposited in the GenBank database using BLAST software. The obtained sequences were phylogenetically analysed with MEGA version 7.0 (Kumar et al. 2016). The dendrogram was prepared with the neighbour-joining method, and the evolutionary distances were calculated with the use of Kimura 2-parameter model (Kimura 1980). To assess the reliability of the constructed phylogenetic tree, the bootstrap test with 1000 replicates was used. A bootstrap value > 75% indicates satisfactory topology of phylogenetic tree branches.

RESULTS

In total, 123 faecal samples and 151 nasal swabs from domestic pigs were surveyed for coronaviruses. The screening with pan-coronavirus primers revealed 12 positive nasal swabs (7.9%), which further tested positive for PHEV with a primer pair targeting a region encoding the hemagglutinin esterase and spike genes. No other members of the family *Coronaviridae* (PEDV, TGEV, PRCV, PDCoV) were detected either in faecal samples or in nasal swabs. The majority of PHEV-positive nasal swabs (n = 9) were found in the group of suckling piglets (zero to four weeks of age) where 29% of tested samples was assessed as positive (n = 9/31). An additional three PHEV-positive samples were detected in the group of fattening pigs (10–24 weeks of age) (3.3%, n = 3/90). Of the eight screened farms, three (37.5%) tested positive for PHEV. All three positive farms were breeding and production farms with closed-herd systems (farm A – 650 gilts and sows, farm B – 1500 gilts and sows, farm C – 800 gilts and sows). The results of PHEV detection in different age categories of pigs and on different farms are summarised in Table 2.

All PHEV-positive samples were further subjected to PCR with a primer pair previously described by Dong et al. (2014) to obtain sequences of a gene segment encoding the nucleocapsid (N) protein (450 aa), which is one of the five main PHEV proteins. The phylogenetic analysis was carried out with the deduced amino acid sequences of the N gene. A nearly complete CDS (444 aa) of the N gene suitable for phylogenetic analysis was

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Table 2. Nasal swabs positive for porcine hemagglutinating encephalomyelitis virus (PHEV) in different age categories of pigs

Farm	Number of PHEV-positive samples (number of samples screened)			
	0–4 weeks	4–10 weeks	10–24 weeks	sows
A	6 (10)	0 (5)	0 (20)	nt
B	3 (17)	0 (5)	0 (4)	0 (6)
C	nt	nt	3 (24)	nt
D	0 (4)	0 (4)	0 (4)	nt
E	nt	0 (10)	nt	nt
F	nt	nt	0 (12)	nt
G	nt	nt	0 (6)	nt
H	nt	nt	0 (20)	nt
Total	9 (31)	0 (24)	3 (90)	0 (6)

nt = not tested

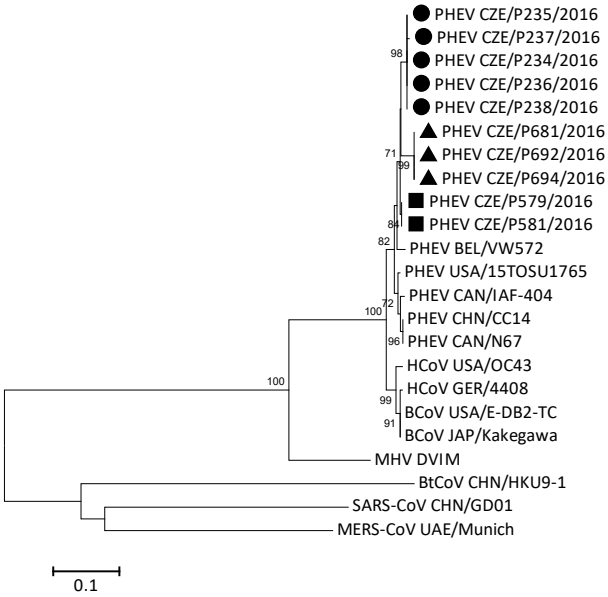

Figure 1. Phylogenetic tree based on the partial coding sequence of the N gene (444 aa) of betacoronaviruses

The Czech porcine hemagglutinating encephalomyelitis virus strains (GenBank MH475347-MH475356) described in this study are marked with a black dot (●), black square (■) or a black triangle (▲) according to the three different sites of collection (farms A, B and C, respectively). GenBank accession numbers of the other analysed strains: DQ011855 (VW572), KY419112 (15TOSU1765), AF481863 (IAF-404), MF083115 (CC14), AY078417 (67N), KF530087 (OC43), FJ415324 (4408), FJ938063 (E-DB2-TC), AB354579 (Kakegawa), AY771998 (DVIM), EF065513 (HKU9-1), AY278489 (GD01), KF192507 (Munich). The tree was generated by the neighbor-joining method using MEGA version 7.0. Bootstrap values (1000 replicates) below 70% were hidden

obtained in nine samples; in sample P235/18 we were able to determine only 352 amino acids which were included in the phylogenetic analysis. All obtained sequences were deposited in the GenBank database with the accession numbers MH475347-MH475356 (P234/16, P235/16, P236/16, P237/16, P238/16, P579/16, P581/16, P681/16, P692/16 and P694/16, respectively). The Czech PHEV strains were compared with the homologous sequences of members of the genus *Betacoronavirus* available in GenBank including other PHEV strains together with bovine and human coronaviruses (BCoV and HCoV), Middle East respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory coronavirus (SARS-CoV), bat coronavirus (BtCoV) and murine hepatitis virus (MHV) to determine their phylogenetic relationship (Figure 1).

The N genes of the Czech PHEV strains showed substantial similarities to PHEV reference strains in GenBank (from 95.8% to 98.1% identity in amino acid sequence and from 95.4% to 97.5% identity in nucleotide sequence). The Czech strains were found to be most similar to the Belgian PHEV strain VW572. Amino acid sequence analysis of the structural N gene revealed non-synonymous substitutions which were identical among PHEV strains collected on the same site but with differences between strains collected from different farms. The number of amino acid substitutions were six (farm B), seven (farm A) and ten (farm C) (Figure 2). The PHEV strains collected from animals on the same farm shared 100% amino acid identity. The identity of amino acid sequences of strains from different farms was 96.7–98.7%. Amino acid identities between the Czech PHEV strains and BCoV and HCoV were quite high and ranged between 94.7% and 95.7%. The phylogenetic analysis also showed no significant homology between the studied PHEV strains and other members of the *Betacoronavirus* genus (BtCoV, MERS-CoV or SARS-CoV).

DISCUSSION

PHEV has been recognised for decades as a cause of serious neurologic disorders of swine. Porcine hemagglutinating encephalomyelitis was reported in several pig-raising countries including Canada

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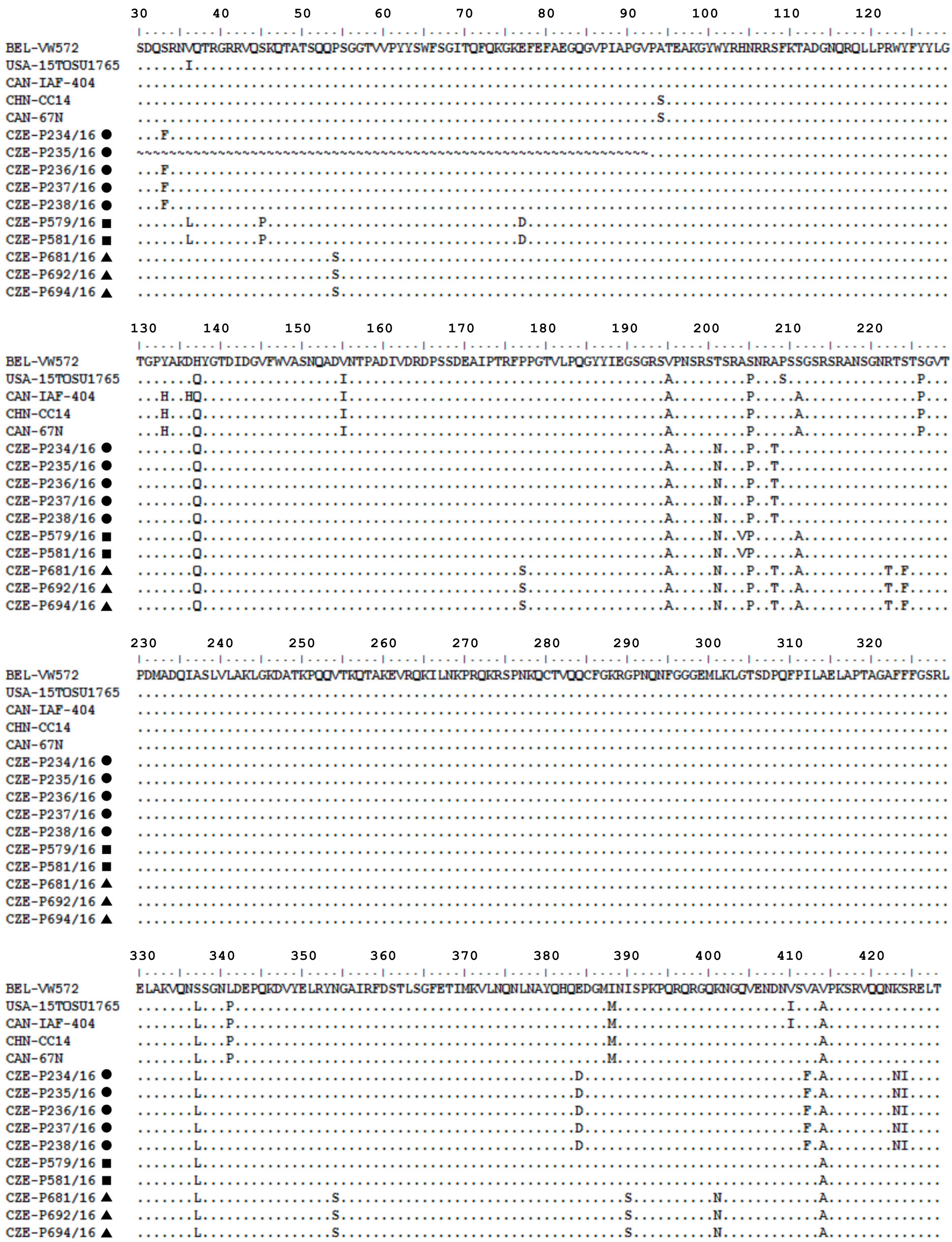

Figure 2. Amino acid sequence alignment including new and some previously described strains of PHEV

Dots represent amino acids identical to reference PHEV strain VW572. Sites of collection are distinguished by different symbols (● = farm A, ■ = farm B, ▲ = farm C)

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(Greig et al. 1962), Belgium (Pensaert and Callebaut 1974), Argentina (Quiroga et al. 2008), China (Gao et al. 2011), South Korea (Rho et al. 2011) and recently in the USA (Lorbach et al. 2017). However, there is no information concerning the prevalence of PHEV in Central Europe and particularly in the Czech Republic. In general, recent data concerning the presence of coronaviruses on Czech pig farms are scarce. Coronavirus-induced porcine epidemic diarrhoea was described in two Czech swine herds at the end of the last century (Smid et al. 1993). Recently, we carried out a serological survey confirming the circulation of PRCV on Czech pig farms (Moutelikova et al. 2016). In this study, a total number of 274 samples was screened for the presence of coronaviruses. The only detected member of the *Coronaviridae* family was PHEV, which was present in 7.9% of tested nasal swabs taken from both suckling piglets (29%, n = 9/31) and fattening pigs (3.3%, n = 3/90). Similarly, Rho et al. (2011), in samples collected from pigs with various clinical symptoms (neurological, respiratory and enteric signs) in South Korea, detected a PHEV prevalence of 9.2% among suckling piglets. In our study, the majority of samples was collected from clinically healthy animals with the exception of one farm where six nasal swabs from suckling one-day-old piglets with acute diarrhoea were analysed and two of them were found to be PHEV-positive. The clinical symptoms of PHEV infection in susceptible animals manifest as CNS disorder, VWD or a combination of both, and may also include diarrhoea (Gao et al. 2011). In our samples, the symptoms were probably not caused by PHEV infection, because the incubation period of VWD or encephalomyelitis after PHEV infection ranges between four and seven days (Pensaert 2006).

Phylogenetic analysis of the N gene of ten Czech PHEV strains showed amino acid sequence identity ranging from 95.8% to 98.1% when compared to other PHEV reference strains in GenBank. Most similar to the Czech PHEV strains was Belgian strain VW572, which was isolated in 1972 from the tonsils of two diseased pigs from a litter where an outbreak of vomiting and wasting disease occurred without further progression towards CNS motoric disorders (Pensaert and Callebaut 1974). Other analysed PHEV strains were isolated either in North America or in China; these strains have evolved separately from the most recent common ancestor which is estimated to have existed as recently as 50 to 60 years ago (Vijgen et al. 2006). Dong et al. (2014) found only two amino acid substitutions in the N gene in Chinese PHEV strains compared to other available PHEV sequences. In the Czech strains the number and position of amino acid changes varied according to the site of collection (seven in farm A, six in farm B, 10 in farm C), which suggests ongoing evolution of the virus. In general, the genome of PHEV strains is largely genetically stable with the spike protein gene being the most variable sequence (Sasseville et al. 2002).

In conclusion, our study demonstrates the presence of PHEV in pigs in the Czech Republic and the genetic characterisation of positive results. To the best of our knowledge, this is the first time PHEV screening was carried out on Czech pig farms. Although no serious outbreak of disease caused by PHEV has been reported in the Czech Republic so far, outbreaks occasionally occur in other countries. Contact with PHEV poses a serious threat especially to serologically naive piglets. Therefore, the endemic presence of PHEV in pig farms and immunisation of piglets via colostral antibodies is very important. Based on the acquired information, the possibility of PHEV infection should be considered in the differential diagnosis of diseases with CNS infection symptoms.

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