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CHARACTERISATION OF HUNGARIAN PORCINE CIRCOVIRUS 2 GENOMES ASSOCIATED WITH PMWS AND PDNS CASES

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The authors report the data of the first survey on the incidence of post-weaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS) in Hungary. A PCR method specific for the detection of porcine circovirus 2 (PCV-2) was developed, which proved to be suitable for diagnostic purposes. PCR screening of organ samples from pigs suspected to be affected with PMWS or PDNS revealed the presence of PCV-2 in 80% of the cases. Six PCV-2 genomes from Hungarian isolates were completely sequenced. Phylogenetic comparison with all the available PCV-2 sequences showed that porcine circoviruses circulating in Hungary are more variable than in several other European countries. Two Hungarian strains clustered together with the Spanish strains forming a distinct group; two others fell in a common group with the French, UK, and Dutch strains, whereas another two strains showed the closest relationship to two of the three known German PCV-2 sequences.

Key words: Porcine circovirus, PMWS, PDNS, Hungary, field strains, virus phylogenetics

Porcine circoviruses (PCV) are small, non-enveloped viruses containing a single-stranded circular DNA genome of approximately 1.76 kb in size. The two types, PCV-1 and PCV-2, described to date share only about 75% nucleotide sequence identity (Mankertz et al., 1997; Meehan et al., 1997) and significantly differ in their pathogenic ability. PCV-1, first detected as a contaminant in porcine kidney cell line PK-15 (Tischer et al., 1974), is considered to be non-pathogenic. Experimental infections of pigs with PCV-1 failed to produce clinical disease in pigs (Tischer et al., 1986; Allan et al., 1995). In contrast, PCV-2,

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which was isolated later, has been identified as a causal infectious agent consistently associated with clinically well-defined diseases.

Postweaning multisystemic wasting syndrome (PMWS) is one of the best characterised diseases of pigs in which the pathogenic role of PCV-2 was demonstrated. Pigs clinically affected with PMWS show growth retardation, dyspnoea, paleness of the skin and moderate to high lethality. Macroscopic and histological lesions include granulomatous interstitial pneumonia with the presence of giant cells, lymphadenopathy and occasionally lymphocytic to granulomatous hepatitis and nephritis. The disease affecting both nursery and fattening pigs was first identified in Western Canada in 1991 (Clark, 1997). Since then, outbreaks of PMWS have been diagnosed in the USA (Daft et al., 1996; Allan et al., 1998; Kiupel et al., 1998), in Asia (Onuki et al., 1999; Choi et al., 2000), and also in European countries including France, Great Britain, Germany, Belgium, Denmark, Italy, Spain, the Netherlands, Northern Ireland, Greece, and Switzerland (Lecann et al., 1997; Ségales et al., 1997; Allan et al., 1998, 1999, 2000; Kennedy et al., 1998; Mankertz et al., 2000; Wellenberg et al., 2000; Borel et al., 2001).

Recently, the presence of PCV-2 has been demonstrated from cases of sow abortion (West et al., 1999; Meehan et al., 2001) and porcine dermatitis and nephropathy syndrome (PDNS), an immune-mediated disorder characterised by systemic vasculitis with marked tropism for the skin and kidney (Rosell et al., 2000; Meehan et al., 2001). In case of both diseases, the pathogenic role of PCV-2 has to be confirmed by further studies. However, among those bacterial and viral agents, which have been suspected to be involved in the pathogenesis of PDNS, PCV-2 is the most likely candidate as aetiologic agent responsible for the development of the syndrome (Allan et al., 2000; Rosell et al., 2000). Although the prevalence of the disease in affected herds is usually low (about 1%), it is a serious concern because the clinical signs (fever, cutaneous haemorrhages) and pathological lesions (petechiae in the renal cortex) closely resemble those of classical swine fever. PDNS was described first in the UK (Smith et al., 1993) then cases were reported from many other countries including Canada, the USA, the Republic of South Africa, Chile, Italy, Northern Ireland, France, Spain, and the Netherlands (Helie et al., 1995; Van Halderen et al., 1995; Ramos-Vara et al., 1997; Sierra et al., 1997; Solignac, 1997; Ségales et al., 1998; Allan et al., 2000; Elbers et al., 2000; Rosell et al., 2000).

The first cases of PMWS and PDNS in Hungary were diagnosed in 1999 (Kiss et al., 2000) and 1997 (Molnár et al., 2002), respectively. Outbreaks of both diseases occur with increasing frequency, and they have become economically important syndromes by now.

In this paper, we report the characterisation of PCV-2 genomes associated with PMWS and PDNS cases in Hungary during 2001 and 2002. The diagnosis was based on clinical history, characteristic pathological and histological lesions,

and detection of the DNA of PCV-2 by PCR. Positive PCV-2 samples were partially sequenced. Based on these partial sequences, we have chosen six viral DNA extracts for complete genome sequencing. The six Hungarian full genomes were compared with PCV-2 sequences available from other countries, and their phylogenetic relationship was analysed.

Materials and methods

Affected animals

During the years 2001 and 2002, 552 35–180-day-old pigs (representing 146 herds and all the 19 counties of Hungary) were submitted by the local veterinarians to the Central Veterinary Institute or to the Department of Pathology and Veterinary Forensic Medicine of the Faculty of Veterinary Science, Szent István University for the confirmation of PMWS and/or PDNS diagnosis. The pigs originated from herds where any of the two diseases was suspected based on clinical history. In a few cases, pigs or blood samples were submitted for screening from farms where no clinical signs were observed.

Gross and histopathological examinations

Pigs were examined post mortem thoroughly and complete necropsy of each animal was performed. Organ samples (from lungs, tonsils, spleen, liver, kidney, mesenteric, inguinal and mediastinal lymph nodes) were collected for histological examination. The samples were fixed in 10% buffered formaldehyde solution and embedded in paraffin, sectioned at 4 μm and stained with haematoxylin and eosin (HE). Samples from the same organs were collected for DNA extraction and kept frozen at –70 °C until processed.

DNA extraction

DNA was extracted from the collected tissue samples separately (by organs) at the beginning of the survey. Later, as the number of samples increased, the organs from one animal were pooled in one tube and extracted together. To 10–15 mg of homogenised tissue, 100 μl TE buffer (10 mM Tris [Tris-(hydroxymethyl)-aminomethane], 1 mM EDTA, pH 8.0), 10 μl of 10% N-lauroylsarcosine (Sigma), 1.4 μl proteinase K enzyme (20 mg/ml; Sigma) were added (in a 1.5-ml Eppendorf tube) and incubated overnight at 55 °C. After incubation, 300 μl of 8 M guanidine HCl (Sigma) and 20 μl of 7.5 M ammonium acetate (Sigma) were added and incubated with shaking at room temperature for one hour. The DNA was precipitated by addition of 1 ml absolute ethanol, then centrifuged at 13,000 rpm for 15 min. The supernatant was discarded and the pellet washed with 75% ethanol, then dried under vacuum (Speed-Vac) for

5 min, and finally resuspended in 40–100 µl of TE buffer (depending on the amount of the DNA). Viral DNA from blood samples was extracted with the same method (kindly provided by Darelle Thompson).

PCR, sequencing and sequence analysis

PCR amplifications in daily routine were performed with PCV-2 specific primers (PCIIF, PCIIR) as described previously by Molnár et al. (2002). From the PCR-positive samples, thirteen were chosen based on different criteria (PMWS and PDNS cases from the same county, cases from geographically distant locations of Hungary, etc.) for partial DNA sequencing. Since primers PCIIF and PCIIR amplify only an approximately 450 base pair (bp) long genome fragment (fragment **a** on Fig. 1), the positive samples were subjected to another PCR yielding an approximately 800 bp long genome fragment (**b** in Fig. 1) with primers PCIIR and PCV-2 S-L Forward (Meehan et al., 2001).

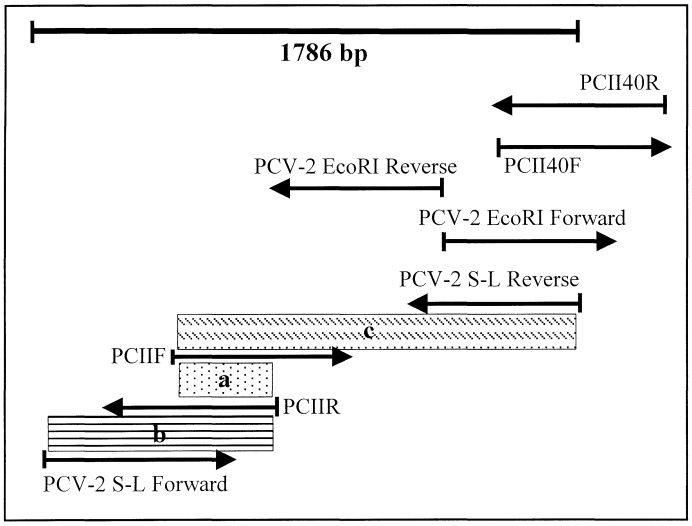

Fig. 1. Schematic presentation of the relative position of the PCR primers and the sequencing strategy of the circovirus genomes. The upper line represents the genome. The primers are indicated by arrows. Boxes indicate genome fragments sequenced with primers used for routine diagnosis (a), partial sequencing (b); or for completion of the genome sequence (c). Arrows longer than the genome length reflect the circular status of virus

Each PCR reaction mixture contained 10 to 100 ng of target DNA in 1 µl volume, 5 µl of REDTaq 10 × PCR buffer (100 mM Tris HCl, pH 8.3, 500 mM KCl, 11 mM $MgCl_2$, and 0.1% gelatine; Sigma-Aldrich), 1 µl of each nucleotide (10 mM; Pharmacia Biotech), 1 µl of both primers (50 pmol/µl), and 2.5 µl of REDTaq DNA polymerase (1 unit/µl; Sigma-Aldrich). The reaction mixture was

adjusted to a final volume of 50 µl by adding MilliQ (Millipore) purified water. The DNA amplification was performed in a programmable thermocycler (PDR-91, BLS, Hungary) with heated cover: 94 °C for 300 sec followed by 35 cycles of 94 °C for 30 sec, 53 °C for 30 sec, and 72 °C for 60 sec. The final elongation step was at 72 °C for 300 sec. The amplification products were analysed by electrophoresis of 10 µl of reaction mixture on 1.0% agarose gel (Sigma-Aldrich) containing ethidium bromide (0.5 µg/ml) at 120 V for 40 min.

For sequencing, the PCR products were purified from agarose gels using a protocol described by Brosius et al. (1996) with a modification of omitting the phenol-chloroform extraction step. Direct cycle sequencing was performed with the same primers used in PCR on an ABI 373A automated DNA sequencer (Applied Biosystems), and the data were processed with the 373A DNA Sequencer Data Analysis Program of Applied Biosystems. Nucleic acid and protein databases were searched using BLASTN and BLASTX (Altschul et al., 1990).

The 13 partial PCV-2 sequences were compared with each other and with all the other available porcine circoviruses from the GenBank using the MultAlin programme (Corpet, 1988). Based on the results, we have selected six of these PCV-2 variants to determine their full genome sequence. For this purpose, a third fragment (**c** in Fig. 1) of approximately 1300 bp amplified with the primers PCIIF and PCV-2 S-L Reverse (Meehan et al., 2001) was purified and directly sequenced with the same PCR primers as well as with two other primers (PCV-2 *Eco*RI Forward and Reverse) that anneal interior to this fragment (Meehan et al., 2001). Finally, an additional primer pair (PCII40F 5'-GACAGTATATCCGAA GGTGC-3' and PCII40R 5'-GTAGACAGGTCACTCCGTTG-3') was designed to amplify an approximately 600-bp-long fragment. The conditions of the PCR (reaction mixture and programme) were identical in each case. The DNA sequences were assembled in one contig using the SeqMan program of the Lasergene package (DNASTAR Inc.).

Phylogenetic analyses were performed with programs included in the PHYLIP (Phylogeny Inference Package, version 3.572c) program package (Felsenstein, 1989). Parallel DNAPARS and DNADIST (with the default 'Kimura 2' parameters) followed by the FITCH application were used for calculations. The validity of the tree topologies obtained by either of the two methods was tested by using bootstrap analysis starting with SEQBOOT with 100 re-samplings from the aligned sequences, followed by parsimony or distance matrix calculations, and the CONSENSE program to calculate the most probable (consensus) tree. The tree was visualised using the program TreeView 1.5.2 (Page, 1996).

Results

Clinical findings and PCR results

Samples from 552 pigs (tonsils, peripheral lymph nodes, liver, kidney, lung and spleen) were submitted for PCV-2 specific PCR, which yielded positive results in 80% of the cases. Considering PCR-positive cases, the most frequent macroscopic findings in their order of prevalence were enlargement of peripheral lymph nodes, emaciation, skin pallor, and serous fluid accumulation in body cavities. Skin lesions characteristic for PDNS had been detected in 59 cases. Lesions characteristic for PMWS and/or PDNS were most frequently encountered in lymph nodes, lungs, kidney and liver. Intracytoplasmic inclusion bodies and giant cells were observed at a relatively low frequency (in 3 and 16% of the PCR positive cases, respectively). The presence of PCV-2-specific nucleic acid was demonstrated in 15% of the cases where no clinical signs or histological findings indicated PCV-2 infection. PCV-2 from blood samples could be demonstrated only during viraemia.

Sequence analyses and phylogenetic calculations

The complete nucleotide sequence of six circovirus genomes was determined by sequencing the PCR-amplified fragments from six field cases. Four of the six genomes had a length of 1768 bp (samples no. 212, 224, 326, and 336) as the majority of PCV-2 variants, while two of them were 1767 bp long (samples no. 304 and 375). The genome organisation proved to be identical with that of the other sequenced PCV-2 variants. The stem-loop structure encompassing the well-conserved nonamer sequence present at the origin of replication of all circoviruses (Meehan et al., 1998) could be identified in all the six Hungarian circovirus sequences, too. The single nucleotide deletion in the genome of samples no. 304 and 375 did not affect the structure of the putative origin of replication. It resulted in an amino acid substitution (K_{232} versus N_{232}) next to the last residue of the putative capsid protein.

The sequences of the six Hungarian variants described in this report were aligned with the other complete PCV-2 genomes available in GenBank (a few identical or very similar non-European strains were omitted) and the alignment was used as input for phylogenetic calculations (Fig. 2).

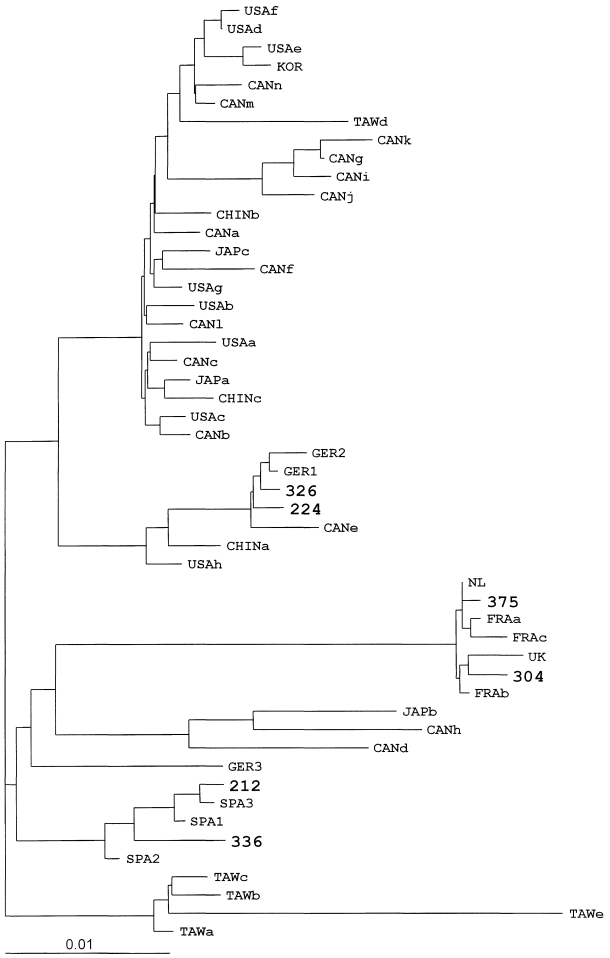

Fig. 2. Phylogenetic tree of complete PCV-2 genome sequences. The unrooted tree was generated by distance matrix analysis (DNADIST, followed by FITCH applying global search option). The strains from Taiwan were chosen as outgroup. The bar represents a divergence of 1% between pairs of neighbouring sequences. Labelling and GenBank accession numbers of the strains are as follows: **Canadian strains:** CANa (AF408635); CANb (AF118095); CANc (AF027217); CANd (AF109398); CANe (AF109399); CANf (AF112862); CANg (AF085695); CANh (AF117753); CANi (AF086835); CANj (AF086834); CANk (AF086836); CANl (AF109397); CANm (AJ293868); CANn (AJ293867); **Chinese strains:** CHINa (AF381176); CHINb (AF381177); CHINc (AF381175); **French strains:** FRAa (AF055393); FRAb (AF055394); FRAc (AF201311); **German strains:** GER1 (AF201305); GER2 (AF201306); GER3 (AF201307); **Japanese strains:** JAPa (AB072303); JAPb (AB072302); JAPc (AB072301); **Korean strain:** KOR (AF454546); **Dutch strain:** NL (AF201897); **Spanish strains:** SPA1 (AF201308); SPA2 (AF201309); SPA3 (AF201310); **Taiwanese strains:** TAWa (AF305533); TAWb (AF154679); TAWc (AF166528); TAWd (AF465211); TAWe (AF364094); **English strain:** UK (AJ293869); **American strains:** USAa (AF147751); USAb (AJ223185); USAc (AF264042); USAd (AF264039); USAe (AF264041); USAf (AF264038); USAg (AF264040); USAh (AF264043); **Hungarian strains:** 212, 224, 304, 326, 336, 375 (AY256455 to AY256460)

Discussion

The first PMWS and PDNS cases in Hungary were diagnosed at the end of the 1990s (Kiss et al., 2000; Molnár et al., 2002). At that time, the occurrence of these syndromes (especially PDNS) was sporadic. However, from the beginning of year 2001, the number of pigs affected with PMWS started to increase significantly. For a few months, cases of PMWS were reported only from one county (Hajdú-Bihar) of Hungary, but later the virus was detected in a rapidly increasing number of counties. In the absence of a comprehensive screening of pig herds for PCV-2 infection, we do not have exact data as to how massive the infection is in Hungary nowadays. However, based on our studies, we can conclude that PMWS was present in every Hungarian county at the end of the year 2001. The diagnosis of PMWS and PDNS was made in all cases on the basis of gross pathological and histological findings and by the specific detection of the DNA of PCV-2 by PCR.

The disease was diagnosed almost exclusively in large pig herds, and only seldom in household pigs. Based on the available case history data and the results of several on-the-spot investigations, clinical signs of the disease occurred most frequently at the age of 45–50 days. The unexpected and significant increase in the number of pigs showing growth retardation, disproportional parts of the body and rough bristles drew attention to the presence of PMWS in a herd. Simultaneously, progressive weight loss was observed in nursery and fattening pigs. In the latest period, more frequent occurrence of respiratory signs was observed among the pigs transferred to the fattening house.

According to our data, the morbidity rate was 10–25% in the vast majority of the cases leading to death or extermination of the diseased pigs. The disease greatly varied on the different farms, lasting from a few weeks up to seven months. On one farm, the clinical signs and the economic losses caused by PMWS persisted for more than a year. The majority of the PMWS cases could be characterised by two patterns of disease progression.

One was characterised by sudden death of 45- to 60-day-old pigs. In these cases, the death of the pigs kept under good conditions was preceded by a relatively short period of weakness, loss of appetite, and rapidly aggravating breathing difficulties. Postmortem examination of these animals characteristically revealed hydrothorax, hydropericardium, and severe lung oedema. In a few cases, the aforementioned lesions were accompanied by acute catarrhal pneumonia localised in the apical and cardiac lobes.

The other form of the diseases was characterised by slower progression and longer persistence. In these cases, the animals showed pallor of skin and mucous membranes, significant weight loss, tachypnoea of different degrees, superficial breathing, and they frequently had greenish-yellowish mucous watery diarrhoea. The lymph nodes were enlarged. The pigs showed these clinical signs for a rela-

tively long period and the majority of them died after 2–3 weeks. Hydrothorax and hydropericardium were more rarely observed at necropsy in this form of the disease. Instead, extensive catarrhal, catarrhal-purulent, occasionally necrotic pneumonia and pleuritis were present. The lesions of the respiratory tract were frequently accompanied by subacute or chronic (in many cases necrotic) enteritis, the enlargement of the mesenteric lymph nodes (resembling a 'string of pearls'), and from mildly icteric to markedly atrophic liver. In such cases, different bacteria (most frequently *Pasteurella multocida*) could be cultured from the lungs.

The clinical signs and pathological findings in the PDNS cases were described in detail in a previous publication (Molnár et al., 2002).

PMWS has been recognised in many countries of Europe, and PCV-2, the supposed aetiological agent of the disease, was studied in detail by methods including DNA sequencing. To date, altogether 11 complete genomes of PCV-2 strains isolated in European countries have been submitted to the GenBank (3 from France, 3 from Spain, 3 from Germany, and 1 from the Netherlands and England each). We were interested to find out the degree of variability of the Hungarian circoviruses and their similarity to other known PCV-2 strains from all over the word. Therefore, from the PCR positive field samples, we chose 13 for partial sequencing. With the exception of two, these samples were chosen from cases (10 PMWS, 3 PDNS) originating from different counties. Sequence comparison of the approximately 800-bp-long nucleotide sequences revealed no similarity among strains from a certain geographical region. The sequence variability could not be linked to the disease manifestation (PMWS or PDNS) either. Therefore, the direction of virus spread in the country or a specific type of PCV-2 causing one of the two diseases could not be established based on simple sequence comparisons or detailed phylogenetic analyses. Similar results were reported by Choi et al. (2002), who have compared seven complete PCV genome sequences (associated with PMWS or congenital tremor) originating from different geographical regions of Indiana State (USA). Nevertheless, based on the reports of veterinary practitioners describing diagnosed outbreaks of these syndromes, expansion of the disease (especially PMWS) from East to West could be observed in the country (Tamás Molnár, unpublished observation). Further data are needed to determine whether this assumption is correct, and why the direction of spread of the disease is not clearly supported by the DNA analyses.

Six strains showing the largest variations in their partial DNA sequence were completely sequenced, thus the number of the known complete European PCV-2 genomes increased by 50%. In case of four viruses the length of the complete genome was identical with most other known PCV-2 genomes, while two of them were shorter by one nucleotide. Such nucleotide deletion was found at the same position in the case of the three French, the Dutch and English strains, too. The English strain has an additional deletion but none of these affect the region thought to be involved in the virus replication.

The Hungarian circovirus sequences described here have the importance of being the first PCV-2 sequences from Central Eastern Europe. On the phylogenetic tree, the Hungarian circoviruses appeared on three different branches. Strains No. 212 and 336 were related to the Spanish strains forming a distinct branch, while strains No. 375 and 304 clustered together with the Dutch, English and the three French strains. Strains No. 224 and 326 were closely related to two German strains. Interestingly, the circoviruses circulating in Hungary seem to be more variable than the known PCV-2 strains in Europe. With the exception of the German strain GER3, other PCV-2 strains originating from a certain country are genetically closely related. Taking into account the degree of the phylogenetic distances, we can assume that the Hungarian PCV-2 strains have different origin. Since the export-import transport of pigs can hardly be followed and there are only a limited number of European PCV-2 DNA sequences deposited in the GenBank to date, an exact epizootiological mapping of the spread of porcine circoviruses in Hungary is not possible yet. However, with the currently quickly increasing number of identified PCV-2 infection cases and that of the fully sequenced PCV-2 variants, a better understanding of the presently puzzling phylogenetic tree can be expected in the near future.

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