DETECTION AND PHYLOGENETIC ANALYSIS OF TYPE 2 PORCINE CIRCOVIRUS (PCV-2) FROM PIGS WITH PMWS IN SLOVENIA, CROATIA AND YUGOSLAVIA

I. Toplak1, Z. Lipej1, T. Petrovic2, P. Hostnik1, J. Grom1, D. Baric-Maganja1
1 Veterinary faculty, Department of Virology, Ljubljana, Slovenia
2 Veterinary Institute, Department of Pathology, Zagreb, Croatia
3 Scientific Veterinary Institute "Novi Sad", Yugoslavia

Key words: PMWS, diagnosis, epidemiology, phylogenetic analysis

Introduction
Porcine circovirus (PCV) has been isolated as a persistent contaminant of porcine kidney cell line PK-15 (1) and no recognized link was found between PCV infection of pigs and disease. Over the last 7 years, a "novel" PCV, designated PCV type 2 (PCV-2) has been associated with various disease syndromes in pigs, primarily postweaning multisystemic wasting syndrome (PMWS), first diagnosed in North America (2), later in many European countries and also some Asian countries. Pigs affected with PMWS showed a variety of clinical signs, including growth retardation, palpable lymphadenopathy, diarrhoea, paleness of skin and dyspnea. At necropsy, the most obvious lesions were marked lymph node enlargement and noncollapsed rubbery lungs. Severe lymphocyte depletion, lymphohistiocytic interstitial pneumonia, hepatitis and nephritis are the most striking histopathologic findings (3). The genomes of PCV-1 and PCV-2 share about 70 % homology (4). On the basis of the complete nucleotide sequences of United States, European and some Asian PCV-2 isolates sequenced thus far, it appears that there exists only one genotype of PCV-2 worldwide. Within the PCV-2 genotype several minor branches that have been identified appear to be associated with geographic origins (5). The U.S. PCV-2 isolates are closely related, but the Canadian isolates vary, to some extent in their genomic sequences. The genomic sequences of three French PCV2 isolates diverge the most from those of other PCV-2. Other branches have also been identified for strain isolated in Taiwan and two of the Canadian PCV2 (5).

In this article, we report about the first genetic comparison of strain isolated in Taiwan and two of the Canadian PCV-2 (5).

Materials and Methods
26 suspected materials for PMWS were tested by PCR. Twenty-two PCR positive samples were included for sequencing: 10 from Slovenia, 7 from Croatia and 5 from Yugoslavia. Lymph node and spleen tissues were kept frozen at -70°C previously to DNA extraction. Total DNA was extracted from positive controls and unknown tissue samples using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Oligonucleotide primers (PCV-2A, PCV2-2B) for the detection of PCV-2 DNAs from laboratory and clinical specimens were used (6). Due to the presence of the PCV-2 in samples, the specific size of PCR product of 501 bp are being amplified. DNA bands of the calculated sizes were excised and recovered from the agarose using Wizard PCR Prep chemistry and spin columns as described by the manufacturer (Promega, UK). The purified PCR products were used as templates in cycle sequencing reactions primed with the PCV2 primers and terminated by fluorescently labelled dideoxynucleotides (BigDye Terminator Cycle Sequencing Kit, PE Biosystems, USA) and analysed as it was described (7), employing the maximum likelihood method of evolution; analysis of 1000 data sets and with the PCV-1 as outgroup.

Results
The phylogenetic tree was constructed from the 453 nucleotides of ORF2. Sequences 1450/01, 181/02, 2186/01 (Slovenia), S-82, S-161 (Croatia), 5/1, 7/1 (Yugoslavia) were generated in this study. Other sequences from Canada, France, Germany, Spain, Netherlands, United Kingdom, China and Taiwan were obtained from the GenBank databases (Figure 1).

Out of the 26 field cases tested by PCR, 22 were PCV-2 positive. The PCR products were sequenced and confirmed to be PCV-2 specific. To characterize the PCV-2 isolates from this study we concentrated on the ORF2 coding region of the viral genome. The homology between 10 Slovenian PCV2, 7 PCV-2 from Croatia and 5 PCV-2 sequences from Yugoslavia in 453 bp of ORF2 was 99.5 %. Phylogenetic analysis of three representative Slovenian (1450/01, 181/02, 2186/01), two Croatian (S-82, S-161) and two Yugoslavian sequences (5/1, 7/1) shows that PCV-2 from our territory were close to published sequences from Netherlands (AF201897), France (AF201311, AF055394, AF055393), China (AY122275), and United Kingdom (AJ293869), (Fig. 1). Phylogenetic analysis of the ORF2 (453 bp), including additional PCV2 sequences from Canada, France, Germany, Spain, Netherlands, United Kingdom, China and Taiwan, taken from the GenBank indicated that these viruses were clustered into at least two phylogenetic groups. The topology of the tree indicates two distinct branches of PCV-2, we were labelled them as subtype PCV-2a and PCV-
2b (Fig 1). Bootstrap value obtained for both suggested subtype was over 95%. The novel subtype PCV2a, comprises PCV-2 from Canada (CAN 1-6), Germany (GER 1-2), Spain (SPA 1-2) and Taiwan (TAI). Isolates from France (FRA 1-3), China (CHI), United Kingdom (UK), Netherlands (NETH) and PCV2 identified from PMWS cases in Slovenia, Croatia and Yugoslavia were clustered together into PCV-2b.

Discussion
To characterise the PCV-2 viruses circulating in the region of Slovenia, Croatia and Yugoslavia, 22 positive samples collected between 2001 and 2003 from pigs suspected for PMWS were subjected to genetic typing. A PCV-2 was first diagnosed from tissues of pigs with PMWS from eight pig farms and five small herds in year 2001 in Slovenia (8), on several finishing pig farms in year 2002 in Croatia (9) and from pigs suspected for classical swine fever/PMWS in year 2003 in Yugoslavia (10). Clinically, PMWS most representative symptoms including wasting, unthriftness, paleness of the skin, respiratory distress and diarrhoea in nursery and fattening pigs were observed (9). A rapid and convenient PCR based test was used for the detection of PCV-2. The 453 bp nucleotide sequence, derived from the ORF2, was aligned and compared to the corresponding positions of published sequences of PCV-2. The phylogenetic analysis of PCV-2 strains analysed in present study showed close relationship to each other and were almost genetically identical with PCV-2 isolated in France, Netherlands, United Kingdom and China. The phylogenetic tree, generated from 453 bp nucleotide sequence comparison, allowed the PCV-2 isolates to be subdivided into at least two clusters, which were labelled as a subtype PCV-2a and PCV-2b. The described method is not only suitable to confirm the presence of PCV-2 in tissue samples of suspected PMWS outbreaks, but also shows genetic differences in ORF2 of PCV-2 isolates from different regions.

References