ISOLATION AND MOLECULAR CHARACTERIZATION OF A PORCINE TESCHOVIRUS 1 ISOLATE FROM CHINA

L. FENG[1], H.Y. SHI[1], S.W. LIU[1], B.P. WU[1], J.F. CHEN[1], D.B. SUN[1], Y.E. TONG[1], M.S. FU[2], Y.F. WANG[1], G.Z. TONG[1]

[1] National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, 427 Ma Duan St., Harbin 150001, P.R. China; [2]Tong Liao Animal Science and Veterinary Research Institute, Inner Mongolia Province 028000, P.R. China

Received February 22, 2006; accepted December 15, 2006

Summary. – Porcine teschovirus 1 (PTV-1) (Swine/CH/IMH/03) was isolated from piglets in a farm in Inner Mongolia Province, P.R. China. It was confirmed by electron microscopy, RT-PCR, and sequencing. Comparison of the sequences of the amino acid and nucleotides and phylogenetic analysis of the polyprotein showed that PTV Swine/CH/IMH/03 strain is PTV-1. The isolated virus has closest relationship with Talfan strain, they shared 98.9% and 99.5% homology of amino acids and nucleotides, respectively, in the ORF of polyprotein. To our knowledge, this is the first report about isolation and identification of a PTV in China.

Key words: isolation; molecular characterization; Porcine teschovirus 1

Introduction

PTVs are causative agents of both severe and mild neurological disorders such as Teschen and Talfan diseases (Harding *et al*., 1957; Trefny, 1930), reproductive failure (Dunne *et al*., 1965), pneumonia (Liebke, 1971), diarrhea (Edington *et al*., 1972), and dermal lesions in swine (Knowles, 1983). The Teschen disease and related mild diseases have been previously called by WHO "porcine transmissible encephalomyelitis". An epidemic of PTV-1 occurred first in central Europe (Teschen, Czech Republic) (Trefny, 1930), and, in the 1950s, spread throughout Europe and caused huge losses to the pig breeding industry. Less severe forms of the disease were first recognized in the United Kingdom in 1956 and was called Talfan disease (Harding *et al*., 1957). Pathogenic and non-pathogenic PTVs have been identified in many countries including USA and Japan (Dunne *et al*., 1967; 1971; Morimoto, *et al*., 1968; Honda *et al*., 1990; Knowles *et al*., 1979), but not in other parts of Asia.

The PTV-1 (the species *Porcine teschovirus*, the genus *Teschovirus*, the family *Picornaviridae*) virion contains a positive-sense single-stranded RNA (ssRNA) of 7.2 kb with a single long ORF. A poly(A) tail is located at the 3'-terminus, while a VPg protein is linked to the 5'-terminus (Honda *et al*., 1990). The species *Porcine teschovirus* contains 11 different viruses (from PTV-1 to PTV-11) (Fanquet *et al*., 2005). Severe and mild nervous clinical symptoms could be caused by different strains/isolates of PTV-1.

To date, only a few molecular studies have been performed on PTVs and their classification to serotypes has depended on biological and biophysical properties (Morimoto *et al*., 1968; Honda *et al*., 1990; Zell *et al*., 2001). Recent recognition of 11 viruses within the species *Porcine teschovirus* relies not only on these properties but also on genomic sequence and organization of the polyprotein gene (Honda *et al*., 1990, Knowles *et al*., 1979; Kaku *et al*., 1999, 2001; Doherty *et al*., 1999; Hyypiä et al., 1997; Zell and Stelzner, 1997).

E-mail: fengli_h@163.com; fax:+86451-82733132.

Abbreviations: PTV-1 = Porcine teschovirus 1

In this study, we isolated PTV-1 from pigs with mild nervous disorders in China and identified them by electron microscopy, RT-PCR, and sequence analysis. To our knowledge, this is the first report on isolation of a PTV-1 in China.

Materials and Methods

Cell lines. Porcine primary kidney cells derived from young pigs and the PK-15 cell line were cultured in the Eagle's Minimum Essential Medium containing 10% of FCS at 37°C.

Virus isolation. Specimens of brain tissues from diseased piglets from a farm in Inner Mongolia Province were used for preparation of a 10% (w/v) pooled suspension in PBS containing 100 U/ml penicillin and 100 mg/ml streptomycin. After 12 hrs at 4°C, 1 ml of the supernatant from the suspension was inoculated onto monolayers of primary cultures of PK-15 cells. After 6 passages in these cells, when CPE reached 80% of population, the cells were harvested, frozen-thaw 3 times, clarified by low-speed centrifugation, and stored at -70°C.

Electron microscopy. Samples of virus-infected cell cultures exhibiting CPE in 80% of population were clarified as above, and the supernatants were centrifuged at 12,000 g for 30 mins. The resulting pellets were resuspended in a minimal volume of deionized water and examined by negative contrast electron microscopy (JEM-1200 EX).

RNA extraction. Two days post infection (p.i.), the cells were mixed with 400 µl of TRIzol Reagent (Gibco BRL) to extract total RNA according to the manufacturer's instructions. The RNA was air-dried and dissolved in 25 µl of RNase-free water.

RT-PCR for amplification of the polyprotein gene of PPV-1 (nt 1752–2895) was employed. In the RT step, total RNA (22 µl) was mixed with oligo$(dT)_{18}$ (50 ng in 4 µl) and incubated at 70°C for 10 mins, followed by 2 mins on ice. Then the reaction mixture (total volume 40 µl) was completed by adding the 5×First Strand Buffer (8 µl), 2.5 mmol/l dNTPs (4 µl), 200 U (1 µl) of RNaseH$^-$ M-MLV reverse transcriptase, and 40 U (1 µl) of RNasin (all from Gibco BRL). After 2 hrs at 37°C, the reaction was terminated by heating at 98°C for 7 mins and chilling on ice.

In the PCR step (Palmquist *et al*., 2002), the reaction mixture (total volume 50 µl) consisted of 15 nmol/l (1 µl) primers CSL1 (5'-CAATACCAAAGGCTGACAT-3', forward, nt 1,869–1,847) and CSL2 (5'-GTGGCTGGAGCATTGATAA-3', (reverse, nt 2895–2876), cDNA (1 µl), the 10×PCR Buffer (5 µl), 2.5 mmol/l dNTPs (4 µl), Taq polymerase (2 µl) (all from TaKaRa, Japan), and water (34 µl). The amplification consisted of 30 cycles of 94°C/1 min (denaturation), 50°C/1 min (annealing), and 72°C/2 mins (extension), followed by final extension at 72°C for 10 mins. The PCR products were detected by 1% agarose gel electrophoresis and ethidium bromide staining.

Cloning, sequencing and sequence analysis. Cloning of PCR products was performed in the T-tailed pMD18-T vector (TaKaRa, Japan). Three clones of each PCR product were sequenced by the dideoxy method. The sequence of the polyprotein gene of PTV-1 Swine/CH/IMH/03 isolate was deposited at the GenBank under Acc. No. DQ355222, and aligned and compared with those of 28 other PTV isolates using the MEGALIGAN Program, Clustual W method, DNAstar version 5.0 (Thompson *et al*., 1994). The following PTV isolates (GenBank Acc. Nos.) were compared: Vir 461/88 (AF 296119), Vir 460/88 (AF 296095), F65 (NC-003985), UKG 173/74 (AF296093), Vir 2899/84 (AF296094), Dresden (AF296096), 21-SZ (AF296117), DS 562/91 (AF296110), F26 (AF296090), F43 (AF296092), O2b (AF296088), PS 36 (AF296089), PS 37 (AF296091), T80 (AF296087), Talfan (AF231769), Teschen-Bozen 654 (AF231767), Teschen-Konratice (AF231768), Vir 918-19/85 (AF296111), Vir 1626/89 (AF296103), Vir 1627/89 (AF296104), Vir 2236/99 (AF296102), Vir 2500/99 (AF296113), Vir 3634/85 (AF2961152), Vir 3764/86 (AF296103), Vir 6711/83 (AF296107), Vir 6793/83 (AF296108), and 25-T-VII (AF296118).

Results and Discussion

To isolate the virus, samples of brain tissues from piglets with mild nervous disorders were inoculated and passaged in PK 15 cell cultures. At the passage 2, the cells showed a CPE characteristic for PTVs, namely rounding, refractility, and lysis. Electron microscopy showed the presence of spherical virus particles of 20 nm in diameter and lacking a lipid envelope at day 2 p.i. (Fig. 1). Using the RT-PCR for amplification of polyprotein gene of PTV-1, a product of about 1.1 kb was detected by agarose gel electrophoresis, while non-infected PK15 cells didn't show any band of that size (Fig. 2).

To confirm the PTV-1 identity of the Swine/CH/IMH/03 isolate, sequence analysis was performed. The isolate was compared with 28 isolates of PTV-1 to PTV-11 from different parts of the world concerning their sequences of polyprotein gene. The results revealed nucleotide and amino acid identities among PTV-1 to PTV-11 of 79.6% to 99.5%, and 88.1% to 98.9%, respectively (Table 1). According to both identities (98.9% and 99.5%, respectively), the Swine/CH/IMH/03 isolate had the closest relationship with PTV-1 Talfan strain (Honda *et al*., 1990). Thus its PTV-1 identity was confirmed. In comparison with the Talfan strain, Swine/CH/IMH/03 contained 36 nucleotide mutations and 24 amino acid substitutions in the ORF for the polyprotein (data not shown). Out of the 36 nucleotide mutations 24 were non-synonymous.

Phylogenetic analysis of 29 PTV isolates/strains showed 11 distinct clusters (Fig. 3), in which Swine/CH/IMH/03 belonged to the same cluster with PTV-1 strains/isolates Talfan, Teschen-Bozen 654, Vir 1626/89, Vir 1627/89, Vir 2236/99, and F65. Further, less related strains/isolates outside of the PTV-1 cluster were Dresden strain (PTV-11), O2b strain (PTV-3), and Vir 460/88 and Vir 461/88 strains (PTV-10).

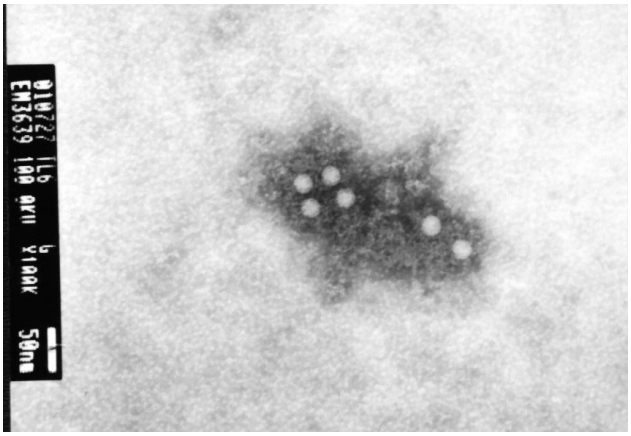

Fig. 1

Electron microscopy of the isolated virus

Magnification 50,000x.

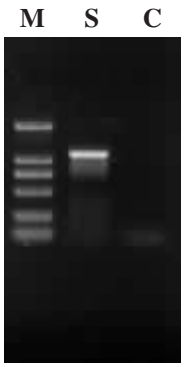

Fig. 2

Agarose gel electrophoresis of RT-PCR products

The isolated virus (lane S), negative control (lane C), and DNA size marker (2000, 1000, 750, 500, 250, and 100 bp) (lane M).

Table 1. Nucleotide and amino acid identities of the polyprotein gene of PTVs

Isolate/ strain[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	95.2	95.9	88.7	92.2	87.9	90.0	88.8	89.5	88.4	90.6	93.4	91.9	94.9	
2	85.2		95.2	90.0	93.6	88.8	89.0	89.9	90.6	88.9	89.4	93.8	91.2	98.9
3	87.5	86.2		89.0	92.6	88.1	89.2	89.2	89.6	88.1	89.6	93.8	90.8	94.9
4	79.8	83.9	80.5		89.7	92.8	92.8	93.7	90.6	92.8	88.0	88.8	88.2	89.6
5	83.4	85.6	83.9	82.0		89.5	89.1	90.4	91.6	88.6	90.4	92.4	92.3	93.2
6	80.3	82.1	80.5	85.4	82.9		92.3	93.0	89.7	91.9	88.2	88.2	87.4	88.4
7	83.3	80.7	81.6	83.8	80.8	83.8		95.1	89.6	92.2	90.1	88.6	90.0	88.6
8	80.5	82.7	81.1	86.1	83.3	86.2	85.3		90.5	92.3	89.0	89.4	88.9	89.4
9	80.6	84.8	80.8	86.1	81.2	83.1	80.1	83.4		89.6	90.0	89.9	89.4	90.1
10	81.0	83.2	80.2	86.3	82.8	84.4	84.4	84.8	84.0		88.0	88.0	88.2	88.4
11	82.6	80.6	81.6	80.1	81.2	80.1	82.6	80.6	81.2	79.2		89.0	90.3	89.2
12	84.6	84.6	85.8	79.6	83.4	80.1	81.3	80.7	80.4	80.4	80.9		90.5	93.4
13	84.7	82.6	83.1	79.8	82.8	79.8	82.0	80.8	79.8	79.0	82.1	81.8		91.0
14	85.1	99.5	86.0	83.8	85.5	82.0	80.7	82.6	84.7	83.1	80.6	84.4	82.6	

Amino acid identity in % (top right); nucleotide identity in % (bottom left).

[a]1 = DS 562/91; 2 = Talfan; 3 = Teschen-Bozen 654; 4 = T80; 5 = O2b; 6 = PS 36; 7 = Vir 3634/85; 8 = PS 37; 9 = F43; 10 = UKG 173/74; 11 = Vir 2899/84; 12 = Dresden; 13 = Vir 461/88; 14 = Swine/CH/IMH/03.

In this study, we isolated PTV-1 from diseased piglets and identified it by electron microscopy, RT-PCR, and sequencing. To our knowledge, this is the first report about isolation and identification of such a virus in China. Although diseases indicating PTVs have often been reported from pig farms, no epidemiological data showing a real epidemic condition in China are so far available,

Acknowledgements. This study was supported by the National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

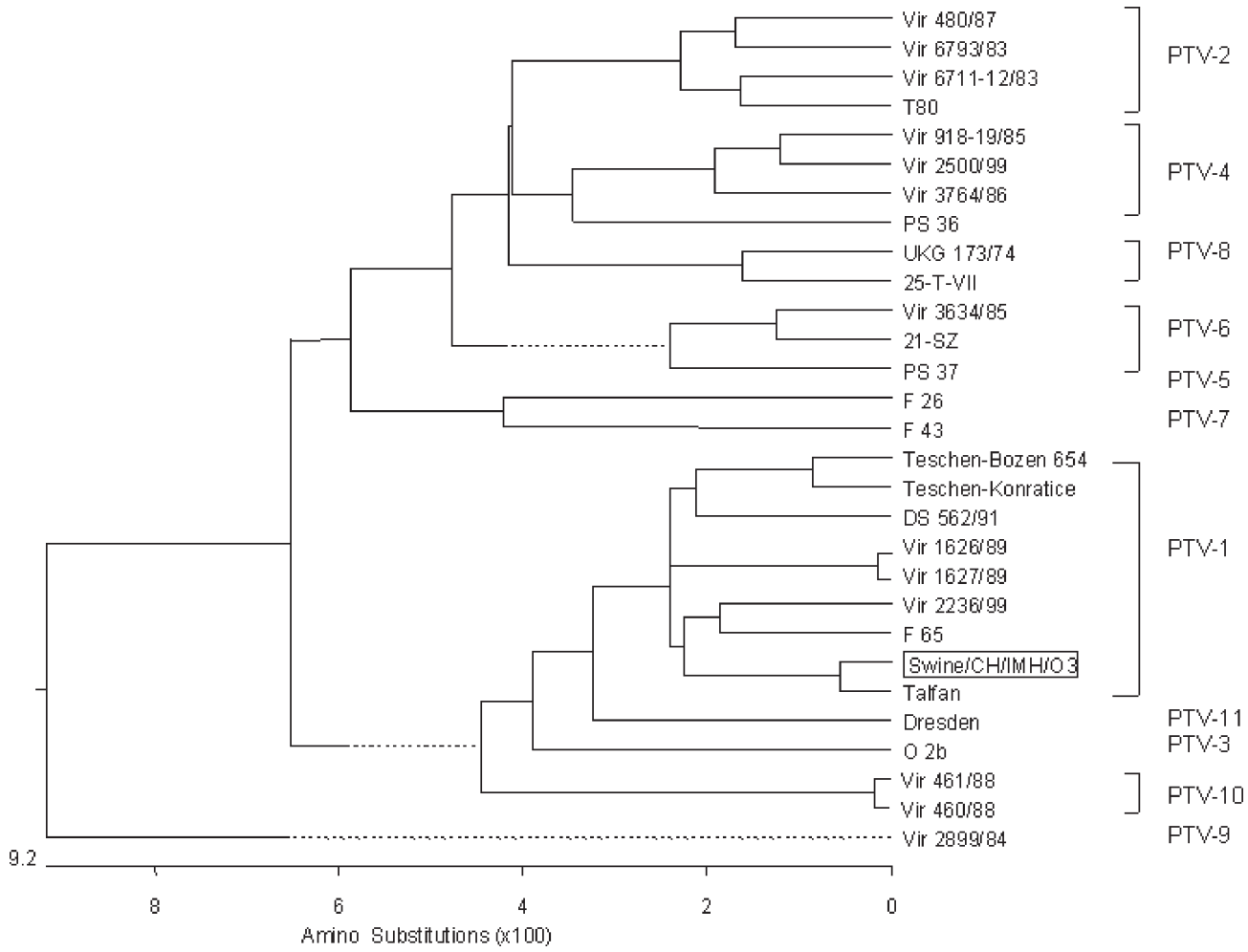

Fig. 3

Phylogenetic tree of PTVs based on the amino acid sequences of polyprotein

References

Alexander TJL, Betts AO (1967): Further studies on porcine enteroviruses isolated at Cambridge. II. Serological grouping. *Res. Vet. Sci.* **8**, 330–337.

Auerbach J, Prager D, Neuhaus S, Loss U, Witte KH (1994): Grouping of porcine enteroviruses by indirect immunofluorescence and description of new serotypes. *J. Vet. Med.* **41**, 277–282.

Doherty M, Todd D, McFerran N, Hoey EM (1999): Sequence analysis of a porcine enterovirus serotype 1 isolate: relationships with other picornaviruses. *J. Gen. Virol.* **80**, 1929–1941.

Dunne HW, Gobble JL, Hokanson JF, Kradel DC, Bubash GR (1965): Porcine reproductive failure associated with a newly identified "SMEDI" group of picornavirus. *Am. J. Vet. Res*. **26**, 1284–1297.

Dunne HW, Wang JT, Ammerman EH (1971): Classification of North American porcine enteroviruses: a comparison with European and Japanese strains. *Infect. Immun*. **4**, 619–631.

Dunne HW, Kradel DC, Clark CD, Bubash GR, Ammermann EH (1967): Porcine enteroviruses: a serologic comparison of thirty-eight Pennsylvania isolates with other reported North American strains, Teschen, Talfan, and T80 serums. A progress report. *Am. J. Vet. Res*. **28**, 557–568.

Edington N, Christofinis GJ, Betts AO (1972): Pathogenicity of Talfan and Konratice strains of Teschen virus in gnotobiotic pigs. *J. Comp. Pathol*. **82**, 393–399.

Fauquet CM, Mayo MA, Maniloff J, Dessellberger U, Ball LA (Eds) (2005): *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses*. Elsevier, Academic Press, Amsterdam, pp. 757–778.

Harding JDJ, Done JT, Kershaw GF (1957): A transmissible polioencephalomyelitis of pigs (Talfan disease). *Vet. Rec*. **69**, 824–832.

Honda E, Kimata A, Hattori I, Kumaga T, Tsuda T, Tokui T (1990): A serological comparison of 4 Japanese isolates of porcine enteroviruses with the international reference strains. *Jpn. J. Vet. Sci.* **52**, 49–54.

Hyypiä T, Hovi T, Nicholas J, Knowles NJ, Stanway G (1997): Classification of enteroviruses based on molecular and biological properties. *J. Gen. Virol*. **78**, 1–11.

Kaku Y, Yamada S, Murakami Y (1999): Sequence determination and phylogenetic analysis of RNA-dependent RNA polymerase (RdRp) of the porcine enterovirus 1 (PEV-1) Talfan strain. *Arch. Virol*. **144**, 1845–1852.

Kaku Y, Sarai A, Murakami Y (2001): Genetic reclassification of porcine enteroviruses. *J. Gen. Viro*l. **82**, 417–424.

Knowles NJ, Buckley LS, Pereira HG (1979): Classification of porcine enteroviruses by antigenic analysis and cytopathic effects in tissue culture: description of 3 new serotypes. *Arch. Virol*. **62**, 201–208.

Knowles NJ (1983): Isolation and identification of porcine enteroviruses in Great Britain, 1979 to 1980. *Brit. Vet. J*. **139**, 19–22.

Liebke H, Schlenstedt D (1971): Eine Enterovirus (ECSO)-Infektion bei Schweinen mit nervösen Störungen und einer gleichzeitig vorhandenen Rhinitis. *Tierärztl. Umschau* **26**, 287–291, 324–330.

Morimoto T, Dunne HW, Wang JT (1968): Serologic comparison of North American and Japanese porcine picornaviruses. *Am. J. Vet. Res*. **29**, 2275–2280.

Palmquist J, Munir S, Taku A, Kapur V, Goyal SM (2002): Detection of porcine teschovirus and enterovirus type II by reverse transcription-polymerase chain reaction. *J. Vet. Diagn. Invest.* **14**, 476–480.

Thompson JD, Higgins DG, Gibson TJ (1994): CLUSTAL W: weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids. Res*. **22**, 4673–4680.

Trefny L (1930): Hromadna onemocnemi vepru na Tesinsku. *Zverolek. Obz*. **23**, 235–236.

Zell R, Stelzner A (1997): Application of genome sequence information to the classification of bovine enteroviruses: the importance of 5´- and 3´-nontranslated regions. *Virus Res*. **51**, 213-229.

Zell R, Dauber M, Krumbholz A, Henke A, Birch-Hirschfeld E, Stelzner A, Prager D, Wurm R (2001): Porcine teschoviruses comprise at least eleven distinct serotypes: molecular and evolutionary aspects. *J. Virol.* **75**, 1620–1631.