Available online at www.sciencedirect.com
ScienceDirect
Virus Research 129 (2007) 104–114
Virus Research
www.elsevier.com/locate/virusres

Sequence analysis of a duck picornavirus isolate indicates that it together with porcine enterovirus type 8 and simian picornavirus type 2 should be assigned to a new picornavirus genus

Chun-Hsien Tseng [a,b], Hsiang-Jung Tsai [a,c,*]

[a] *Graduate Institute of Veterinary Medicine, College of Bioresource and Agriculture, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan*
[b] *Division of Bio-production, National Institute for Animal Health, Council of Agriculture, 376 Chung-Cheng Road, Tamsui, Taipei 251, Taiwan*
[c] *Center for Zoonoses Research, College of Bioresource and Agriculture, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan*

Received 2 March 2007; accepted 26 June 2007
Available online 7 August 2007

Abstract

In a 1990 outbreak, a virus isolated in Taiwan from the intestines of ducks showing signs of hepatitis was tentatively classified as a picornavirus on the basis of physical, chemical, and morphological characteristics. The virus was cloned and then found not to be type 1 duck hepatitis virus (DHV-1) or a new serotype of duck hepatitis virus (N-DHV) by serum neutralization. Complete genome sequencing indicated that the virus genome had 8351 nucleotides and the typical picornavirus genome organization (i.e., 5′ untranslated region (UTR)-L-P1 (VP 4-2-3-1)-P2 (2A-B-C)-P3 (3A-B-C-D)-3′ UTR-poly A). One open reading frame encoded 2521 amino acids, which makes this virus one of the largest picornaviruses, second only to equine rhinitis B virus of the genus *Erbovirus*. Its L protein was the largest within the family *Picornaviridae* (451 amino acids) and suspected to be a trypsin-like protease. The 235-nucleotide 3′ UTR region was of intermediate size, quite long compared to other picornaviruses but shorter than other picornaviruses of duck-origin (DHV-1 and N-DHV) and had four regions of secondary structure. The 2A protein was composed of only 12 amino acids, which is the shortest of any member of the family *Picornaviridae*. Phylogenetic analysis of the polyprotein and 3D sequences indicated that this virus (named duck picornavirus [DPV]) together with porcine enterovirus type 8 virus and several simian picornaviruses form a distinct branch of the family *Picornaviridae* and should be assigned to a new picornavirus genus.

Keywords: Duck picornavirus; DPV; Genome analysis; *Picornaviridae*

1. Introduction

In 1990, several duck picornaviruses were isolated from a co-outbreak of duck parvovirus and a new serotype of duck hepatitis virus (N-DHV) in Taiwan (Lu et al., 1993; Tseng and Tsai, 2007). A duck picornavirus (DPV strain TW90A) that was isolated from the intestinal tracts of ducklings with duck virus hepatitis (DVH) could not produce the typical hemorrhagic lesions of hepatitis and was not as deadly in day-old ducklings. The results of cross-neutralization testing indicated that DPV TW90A was not antigenically related to type 1 duck hepatitis virus (DHV-1) or to N-DHV. Sequence analysis also indicated that DPV differed from DHV-1 and N-DHV in sequence and genome organization. Nevertheless, DPV, porcine enterovirus serotype 8 (PEV-8), and several simian picornaviruses (SV-2, 16, 18, 42, 44, 45, and 49) had a high percentage of sequence identity and shared the same genome organization.

Porcine enteroviruses (PEVs) have been placed in the genus *Enterovirus* and divided into 11 serotypes (PEV-1–11) on the basis of virus neutralization data (Knowles et al., 1979). At least 15 serotypes have been identified to date. Furthermore, these serotypes have also been divided into three groups (I–III) based upon the type of cytopathic effect (CPE) produced in pig kidney cells. PEV-8 was at first assigned to group II (Kaku et al., 2001)

* Corresponding author at: Graduate Institute of Veterinary Medicine, College of Bioresource and Agriculture, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan. Tel.: +886 2 3366 3861; fax: +886 2 2364 9154.
E-mail address: tsaihj@ntu.edu.tw (H.-J. Tsai).

0168-1702/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2007.06.023

but later reclassified as a member of the enterovirus species *PEV-A* by the Picornavirus Study Group (Krumbholz et al., 2002). The simian picornaviruses (SVs) were isolated from various primate tissues in the 1950–1970s, and 20 simian picornavirus serotypes have been recognized. SVs were once classified in the genus *Enterovirus*, but evolutionary analysis of the VP1 nucleotide sequence of SVs revealed that SV-2, SV-16, SV-18, SV-42, SV-44, SV-45, and SV-49 are clearly distinct from members of the *Enterovirus* genus and from other existing picornavirus genera. These serotypes of SVs are called SV-2-like virus and appear to represent a new picornavirus genus (Oberste et al., 2002).

Among *Picornaviridae* family, PEV-8, and SV-2-like viruses are most similar to the *Enterovirus* and *Rhinovirus*, especially in their 3D region proteins, which have amino acid sequence identities higher than 50%. But PEV-8 and SV-2-like viruses have an L protein at the N-terminus of the polyprotein that is lacking in the *Enterovirus* and *Rhinovirus.* Also the size of the 2A proteins of PEV-8 and SV-2-like viruses (226 and 302 amino acids, respectively) is much longer than the 136–150 amino acids of the *Rhinovirus* and *Enterovirus* (Krumbholz et al., 2002; Oberste et al., 2002, 2003).

Several features of the DPV are similar to those of PEV-8 and SV-2-like viruses: (1) an L protein in the polyprotein N-terminal and (2) amino acid sequence percentage identity in the 3D region (DPV to PEV-8 and SV-2-like virus similarities are as high as 56.4% and 62.4%, respectively). Moreover, the results of the evolutionary analysis indicated that DPV together with PEV-8 and SV-2-like viruses belonged to a new genus that differs from *Rhinovirus* and *Enterovirus.* The Picornavirus Study Group recently proposed the establishment of a new "Sapelovirus" genus in the *Picornaviridae*, and the inclusion of three species: Porcine enterovirus A (PEV-8) ("Porcine sapelovirus"), SV-2-like viruses ("Simian sapelovirus") and duck picornavirus TW90A ("Avian sapelovirus") (http://www.picornastudygroup.com/proposals/2006/proposals_2006.htm).

2. Materials and methods

2.1. Virus growth and characterization

Virus growth curves of DPV were obtained by infecting monolayers of primary duck kidney (DK) cells in 60-mm plastic Petri dishes with 0.5 ml of DPV at a multiplicity of infection of 1.0 (10^4 $TCID_{50}$). After a 1-h virus adsorption period, the inoculum was removed, and cultures were washed three times with 5 ml of MEM. Cultures then received 5 ml of MEM, and incubation was continued at 37 °C. At selected intervals after infection, samples were withdrawn for infectivity assay of the released virus. DK cells were also harvested to determine the virus titre. Hyperimmune serum against DPV was produced in SPF chickens and was used to detect DPV in the infected DK cells by an indirect fluorescence assay.

DK cells infected with DPV were treated with 0, 0.01, or 0.05 μg/ml of actinomycin D, respectively, and were titrated at 24 and 48 h after treatment to determine the sensitivity of the replication of the DPV to actinomycin D. The sensitivity of the DPV to a lipid solvent (chloroform), pH, and heat were also examined using previously described methods (Tseng and Tsai, 2007).

2.2. Pathogenicity test

Day-old DPV-antibody negative Tsai-Ya ducklings were infected with DPV *via* oral, intramuscular, intravenous, or intraperitoneal routes. The dose of each inoculation was $10^{6.5}$ 50% tissue culture infectious dose ($TCID_{50}$) per bird. The inoculated ducklings were observed for 1 week. The mortality rate was recorded and the body weight difference between the DPV-inoculated group and the non-inoculated control group were determined.

2.3. Production of the hyperimmune sera

Hyperimmune sera against DPV (strain TW90A) were produced in day-old SPF chicks maintained at the National Veterinary Institute, Council of Agriculture, Taiwan, by previously described methods (Tseng and Tsai, 2007). Briefly, the birds were immunized once by the oral route and subsequently three times by the intramuscular route at weekly intervals. The dose of each immunization was $10^{8.4}$ $TCID_{50}$ per bird. The hyperimmune serum was inactivated at 56 °C for 30 min.

2.4. Cross-neutralization tests

Cross-neutralization tests were performed on DK cells by the constant virus-variable serum method, to determine the antigenic relationship between DHV-1 (strain 03D), N-DHV (strain 90D), and DPV (strain TW90A) (Tseng et al., 2007; Tseng and Tsai, 2007).

2.5. Purification of duck picornavirus TW90A

DPV (strain TW90A) was first concentrated 1000-fold by 20% (w/v) ammonium sulphate precipitation and then subjected to 10–40% caesium chloride gradient centrifugation at 100,000 × *g* for 22 h. The virus band was collected and diluted with a proper amount of D_2W and centrifuged at 100,000 × *g* for 3 h. The pellet was dissolved in 1/1000 of the original volume of D_2W. The virion RNA was extracted using the QIAamp® Viral RNA Mini Kit (Qiagen Ltd., Hilden, Germany) and was used in the following study.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) and sequencing of DPV

The MegAlign program (Clustal W method) (Lasergene® expert sequence analysis software version 5, DNASTAR Inc., Madison, WI, USA) (Thompson et al., 1994) was used to align nucleotide and amino acid sequences of representative strains from nine virus genera in the *Picornaviridae* and the two candidate "sapeloviruses". Degenerate PCR primers were designed for the 3CD gene region based on conserved motifs in picornaviruses of different genera. A BLAST (Basic Local Alignment Search Tool; National Center for Biotechnology Information)