

COMPARISON OF NUCLEOTIDE SEQUENCE OF p2C REGION IN DIABETOGENIC AND NON-DIABETOGENIC COXSACKIE VIRUS B5 ISOLATES

Cheng-Chong Chou, Kuei-Hsiang Lin, Guan-Ming Ke, Yi-Ching Tung, Mei-Chyn Chao,¹
Jeng-Yin Cheng, and Bai-Hsiun Chen

Departments of Laboratory Medicine and ¹Pediatrics, School of Medicine,
Kaohsiung Medical University, Kaohsiung, Taiwan.

Enteroviruses are environmental triggers in the pathogenesis of type 1 diabetes mellitus (DM). A sequence of six identical amino acids (PEVKEK) is shared by the 2C protein of Coxsackie virus B and the glutamic acid decarboxylase (GAD) molecules. Between 1995 and 2002, we investigated 22 Coxsackie virus B5 (CVB5) isolates from southern Taiwan. Four of these isolates were obtained from four new-onset type 1 DM patients with diabetic ketoacidosis. We compared a 300 nucleotide sequence in the 2C protein gene (p2C) in 24 CVB5 isolates (4 diabetogenic, 18 non-diabetogenic and 2 prototype). We found 0.3–10% nucleotide differences. In the four isolates from type 1 DM patients, there was only 2.4–3.4% nucleotide difference, and there was only 1.7–7.1% nucleotide difference between type 1 DM isolates and non-diabetogenic isolates. Comparison of the nucleotide sequence between prototype virus and 22 CVB5 isolates revealed 18.4–24.1% difference. Twenty-one CVB5 isolates from type 1 DM and non-type 1 DM patients contained the PEVKEK sequence, as shown by the p2C nucleotide sequence. Our data showed that the viral p2C sequence with homology with GAD is highly conserved in CVB5 isolates. There was no difference between diabetogenic and non-diabetogenic CVB5 isolates. All four type 1 DM patients had at least one of the genetic susceptibility alleles HLA-DR, DQA1, DQB1. Other genetic and autoimmune factors such as HLA genetic susceptibility and GAD may also play important roles in the pathogenesis in type 1 DM.

Key Words: nucleotide sequence, Coxsackie virus B5, type 1 diabetes mellitus
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Type 1 diabetes mellitus (DM) results from the progressive destruction of pancreatic beta cells. Environmental factors are believed to play an important role in the development of type 1 DM by influencing the penetrance of diabetes susceptibility genes. Viruses, especially enteroviruses, have long been considered to play a part in this disease [1–3], based on several serologic and epidemiologic studies [4,5].

Diagnostic tests for enteroviruses include viral isolation, immunologic methods and the polymerase chain reaction

(PCR). Traditionally, viral isolation in cell culture is the gold standard of enterovirus diagnosis. However, diagnosis by cell culture is relatively slow, requires significant technical expertise and may not succeed for certain enterovirus serotypes and/or certain clinical situations. Immunologic methods based on antigen or antibody detection have been limited by the absence of a single common antigen among the enteroviruses [6].

Very few reports have described viral isolation associated with the development of type 1 DM. In 1979, Yoon et al first reported the isolation of a virus from the pancreas of a child with diabetic ketoacidosis [7]. In 1982, Champ-saur et al reported isolation of a Coxsackie virus from a child dying of type 1 DM, and demonstrated that these isolated viruses could induce diabetes in mice [8]. However, there

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Address correspondence and reprint requests to: Dr. Bai-Hsiun Chen, Department of Laboratory Medicine, Kaohsiung Medical University Hospital, 100 Tzyou 1st Road, Kaohsiung 807, Taiwan.
E-mail: chen_bh.tw@yahoo.com.tw

are several reports that viral isolates do not induce diabetes in mice [9–11].

During the period 1995–1998, we investigated the association between Coxsackie virus infection and type 1 DM through viral isolation and serologic methods. We found four Coxsackie virus B5 (CVB5) isolates in throat swabs or stool specimens from 38 consecutive new-onset type 1 DM patients admitted with diabetic ketoacidosis or ketosis. There was an epidemic of CVB5 infection during that period in southern Taiwan. All four of the children with CVB5 isolates suffered from type 1 DM, while the other children suffered from aseptic meningitis, pneumonia or other diseases. We were interested in whether there was a significant difference in nucleotide sequence between diabetogenic and non-diabetogenic CVB5 isolates.

The 2C protein is a non-structural protein with large-ly unknown function. It is apparently required in the initiation and elongation phases in RNA synthesis [12]. A sequence of six identical amino acids is shared by the 2C protein of Coxsackie virus B4 and the 65 kDa glutamic acid decarboxylase (GAD65) molecule, which is a major autoantigen in type 1 DM (so-called molecular mimicry) [13–19]. Studies analyzing the possible role of molecular mimicry in type 1 DM have been largely focused on this sequence [20].

The main purposes of this study were to find the differences in the nucleotide sequence of the gene encoding the 2C protein (p2C), and to compare the frequency of the amino acid PEVKEK sequence, between diabetogenic and non-diabetogenic CVB5 isolates.

MATERIALS AND METHODS

Between 1995 and 2002, we randomly investigated 22 CVB5 isolates in southern Taiwan. Twenty CVB5 isolates were obtained from the Division of Virology, Department of Laboratory Medicine, Kaohsiung Medical University (KMU) Hospital. The other two CVB5 isolates were kindly provided by Kaohsiung Chang-Kang Hospital. Four of the 22 CVB5 isolates came from four children with type 1 DM. These four CVB5 strains were isolated from 38 consecutive throat swabs or rectal/stool specimens collected, within 4 days after admission, from new-onset type 1 DM patients admitted with ketoacidosis between 1995 and 1998. All four type 1 DM patients suffered from symptoms such as polyuria, polydipsia, polyphagia, fever, vomiting or abdominal pain several days prior to their admission. Three of these patients had blood pH values

less than 7.3 at admission.

Human leukocyte antigen (HLA)-DR and HLA-DQA1, DQB1 studies were performed using the sequence-specific primer methods developed by Olerup et al [21,22]. All CVB5 isolates were identified using a neutralization test with type-specific antiserum against the prototype strain of CVB5. HeLa cells were grown in culture with Hanks minimum essential medium supplemented with 10% calf serum, and used to isolate viruses from throat swabs and stool specimens. After complete cytopathic effect (CPE) was reached, the cultures were frozen and thawed three times.

RNA was extracted using a single extraction procedure [23]. Reverse transcription-PCR was performed as previously described [24]. Briefly, cDNA was synthesized in a 20 μ L reaction mixture containing 75 mmol/L KCl, 50 mmol/L Tris-HCl pH 8.3, 3 mmol/L MgCl₂, 10 mmol/L dithiothreitol, 0.2 mmol/L of each dNTP (Promega Corp, Madison, WI, USA), 50 pmol of the denatured antisense primer (5'-TCTTCACCGTGGCGGTGGCTCAAACA-3'), 5 units of avian myeloblastosis virus reverse transcriptase (Promega Corp) and extracted RNA. After incubation at 37°C for 60 minutes, 80 μ L of the PCR mixture was added. The PCR mixture contained 50 mmol/L KCl, 10 mmol/L Tris-HCl pH 8.9, 3.6 mmol/L MgCl₂, 0.2 mmol/L of each dNTP, 100 μ g/nL bovine serum albumin, 80 pmol of sense primer (5'-GCATTGGACTTGACTGTATG-3'), 40 pmol of antisense primer (5'-TCTTCACCGTGGCGGTGGCTCAAACA-3'), and 0.2 units of Taq DNA polymerase (Promega Corp).

RNA-cDNA hybrids were denatured at 94°C for 5 minutes. Amplification was performed in 40 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 58°C for 30 seconds, and elongation at 72°C for 50 seconds. The reactions were analyzed by electrophoresis at 1.5 v in agarose gels, and the product was purified from low-melting point agarose with a DNA purification system (Promega Corp), according to the manufacturer's instructions. DNA sequence analysis was performed using the Ampli Cycle sequence kit, according to the manufacturer's instructions, in an automated sequencer (ABI PRISM 310; Perkin Elmer, Foster City, CA, USA).

A 300 nucleotide sequence of p2C in 22 CVB5 isolates was compared with that of two published CVB5 prototypes (gi-59045 and gi-6650682) using nucleotide comparison software (NCBI-Blast and DNASTAR-John Hein methods).

Accession numbers of the nucleotide sequences

The p2C nucleotide sequences of the 22 CVB5 isolates

reported in this paper appear in the GenBank nucleotide sequence database with the following accession numbers: 522/95, AY738592; 855/95, AY744161; 892/95, AY744162; 811/97, AY744163; 677/95, AY744164; 790/95, AY744165; 851/95, AY744166; 852/95, AY744167; 886/95, AY744168; 889/95, AY744169; 894/95, AY744170; 059/96, AY744171; 064/96, AY744172; 068/96, AY744173; 072/96, AY744174; 080/96, AY744175; 081/96, AY744176; 1214/9, AY744177; 212/96, AY744178; 524/98, AY744179; 0090/98, AY744180; 3203/98, AY744181.

RESULTS

Most specimens of the CVB5 isolates were obtained from throat swab and stool specimens (Table 1). Only one CVB5 isolate came from cerebrospinal fluid in an aseptic meningitis patient. All CVB5 isolates reached CPE in HeLa cells. There was no significant difference in mean time to CPE between diabetogenic and non-diabetogenic CVB5 isolates. Table 2 shows the demographic characteristics and laboratory results for the four type 1 DM patients.

Table 1. Clinical characteristics and specimen source of 22 Coxsackie virus B5 isolates

Isolate	Specimen	Diagnosis	CPE, days	Month isolated
KMU95-522	Rectal swab	Type 1 DM	2	1995/10
KMU95-677	Throat swab	URI, CHD	3	1995/11
KMU95-790	Stool	AGE	6	1995/12
KMU95-851	Throat swab	Pneumonia	6	1996/01
KMU95-852	Stool	Viral meningitis	4	1996/01
KMU95-855	Throat swab	Type 1 DM	4	1996/01
KMU95-886	Throat swab	Viral meningitis	4	1996/01
KMU95-889	Throat swab	Pneumonia	5	1996/01
KMU95-892	Throat swab	Type 1 DM	2	1996/01
KMU95-894	Throat swab	Pneumonia	4	1996/01
KMU96-059	Throat swab	Pneumonia	5	1996/01
KMU96-064	Throat swab	Sepsis	5	1996/01
KMU96-068	Throat swab	Viral meningitis	3	1996/01
KMU96-072	Stool	Fever	5	1996/01
KMU96-080	Throat swab	Pneumonia	4	1996/02
KMU96-081	Stool	AGE	4	1996/02
KMU96-1213	Throat swab	Acute bronchitis	2	1996/09
KMU96-212	Throat swab	Status asthmaticus	2	1996/03
KMU97-811	Throat swab	Type 1 DM	4	1997/12
KMU98-524	CSF	Viral meningitis	4	1998/07
KCK98-090	Throat swab	Viral encephalitis	NA	1998
KCK02-3203	Rectal swab	AGE, seizure	NA	2002

CPE = complete cytopathic effect; DM = diabetes mellitus; URI = upper respiratory tract infection; CHD = congenital heart disease; AGE = acute gastroenteritis; CSF = cerebrospinal fluid; NA = not available.

Table 2. Demographic characteristics and laboratory results of four type 1 diabetes mellitus patients

Case	Gender/Age, yr	Duration, yr	GAD	HLA			Serology	
				DR	DQA1	DQB1	Acute	Conv
1	F /5	4	Neg	2, 4	0103, 0302	0401, 0602	16	32
2	F /14	5	50.9	3, 4	0301, 0501	0201, 0401	4	NA
3	M /1	2	Neg	4, 9	0301, 0301	0201, 0301	32	NA
4	M /6	3	7.1	3, 11	0501, 0501	0201, 0301	8	NA

GAD = glutamic acid decarboxylase; HLA = human leukocyte antigen; DR = D-related; Conv = convalescence; F = female; Neg = negative; NA = not available; M = male.

for PEVKEK, which is also conserved in the nucleotide sequence of GAD, the main autoantigen of type 1 DM.

Comparisons of this 300 nucleotide sequence in 22 diabetogenic or non-diabetogenic CVB5 isolates and two prototype CVB5 isolates revealed 0.3–10% nucleotide difference. Among the four CVB5 isolates from the type 1 DM patients, there was only 2.4–3.4% nucleotide difference, and there was only 1.7–7.1% nucleotide difference between these type 1 DM isolates and the non-diabetogenic CVB5 isolates. The CVB5 isolate KMU98-524 had the greatest nucleotide difference from other CVB5 isolates (5.2–10%). Comparison of nucleotide sequences between prototype isolates and these 22 CVB5 isolates revealed 18.4–24.1% difference (Figure).

DISCUSSION

Diseases with seasonal incidence are often caused by viral infections. There have been many anecdotal reports of a viral infection preceding or coinciding with the onset of type 1 DM [25], as well as case reports of virus isolation from the pancreas of patients who have died of acute diabetes, and reports of induction of diabetes in susceptible animals by infection with isolated viruses [7]. In this study, most CVB5 strains were isolated in the winter.

Kopecka et al found that CVB5 isolates from the same outbreak were closely related, not exceeding 7.2% nucleotide difference, and that the differences were greater between isolates from different outbreaks, varying between

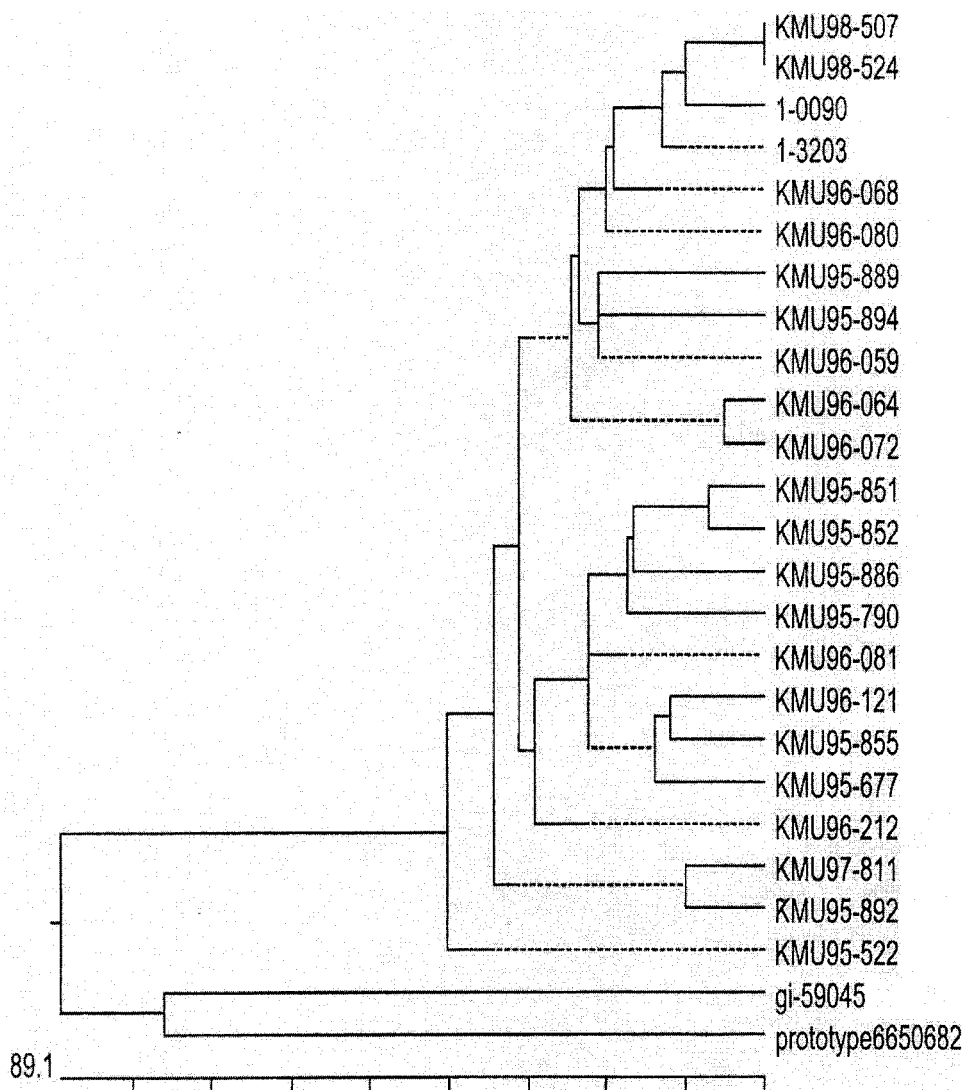


Figure. Dendrogram illustrating sequence relationships among the 22 Coxsackie virus B5 isolates and two published prototypes.

8.4% and 16% [26]. Their studies also revealed that CVB5 from an outbreak in 1967 was more similar to viruses from an outbreak in 1983 than to those isolated from an intervening outbreak in 1972. The differences in CVB5 nucleotide sequences in Kopecka et al's study (< 7.2%) were similar to those reported in this study (0.3–10%).

To date, 13 different viruses have been reported to be associated with the development of type 1 DM in humans and in various animal models, including Coxsackie virus B (CVB) [27], rubella virus [28], mumps virus [29], cytomegalovirus [30], Epstein-Barr virus and varicella zoster [31]. In animals, nine viruses have been reported to be associated with the development of type 1 DM [32].

In this study, all CVB5 isolates contained the PEVKEK sequence. Vreugdenhil et al determined the amino acid sequence 1129–1154 of 10 different CVB4 and 11 different CVB-like enteroviruses [20]. They showed that the viral p2C sequence with homology to GAD is highly conserved in CVB-like enteroviruses. In their study, within the PEVKEK motif, a single amino acid substitution of lysine to arginine (K–R) was observed in two of 10 CVB4 isolates. They also demonstrated that when these CVB-like enterovirus sequences were compared to the published CVB4 sequence, the homology of 100 nucleotides in the p2C gene was 79% (74–85%). There was 76–82% nucleotide sequence homology in our CVB5 isolates when compared with prototype CVB5 isolates.

In this study, almost all p2C nucleotide sequences in diabetogenic and non-diabetogenic CVB5 isolates contained PEVKEK, which was conserved in the nucleotide sequence of GAD. In animal studies, Ju and Yoon [33] and Chung et al [34] demonstrated that Kilham rat virus (KRV)-induced autoimmune diabetes in diabetes-resistant BB rats is not due to molecular mimicry, but to a breakdown of the finely tuned immune balance of Th1-like CD45RC+CD4+ and Th2-like CD45RC-CD4+ T cells, resulting in selective activation of beta cell-cytotoxic effector T cells.

Two of four type 1 DM patients were positive for GAD65 in this study, which was similar to our GAD studies of seropositivity (54.3%) in type 1 DM patients in Taiwan [35]. Tests for GAD65 were performed several years after admission in the studies of Chuang et al [36] and Falorni et al [37], who observed that GAD65 positivity decreased as the diabetes duration increased. However, our results revealed that GAD seropositivity did not decrease as the duration of type 1 DM increased [35].

In this study, the HLA-DR assays revealed that the four type 1 DM patients had at least one susceptible HLA-DR allele, such as DR3, DR4 and DR9, known in Chinese child-

onset type 1 DM [36,38]. Our HLA-DQA1, DQB1 studies showed that the four type 1 DM patients also had at least one genetic susceptibility allele, such as HLA-DQA1 0301, 0302, DQB1 0201 [39]. Two of these patients had the susceptible haplotype DQA1 0301, DQB1 0201, with a relative risk of 42.6. A third patient had the susceptible haplotype DQA1 0501, DQB1 0201, with a relative risk of 6.6 [39].

In this study, all CVB5 isolates contained the PEVKEK sequence, whether isolated from type 1 DM or non-diabetic patients. Our data showed that the viral p2C sequence with homology to GAD is highly conserved in CVB5 isolates. There was only 2.4–3.4% nucleotide difference in our four isolates from type 1 DM patients and only 1.7–7.1% nucleotide difference between the type 1 DM and non-diabetes CVB5 isolates. Other genetic and autoimmune factors such as HLA genetic susceptibility and GAD may also play important roles in the pathogenesis of type 1 DM.

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引致與非引致糖尿病之克沙奇 B5 病毒株其 p2C 核酸序列之比較

周正忠¹ 林貴香¹ 董宜青¹ 柯冠銘¹ 趙美琴² 鄭貞英¹ 陳百薰¹

高雄醫學大學醫學院 ¹實驗診斷學 ²小兒科

腸病毒是導致第一型糖尿病病因之環境促發因子，B 群克沙奇病毒其 2C 蛋白中有六個胺基酸序列 (PEVKEK) 與第一型糖尿病之主要自體免疫抗原：麩胺酸脫羧醯素 (65GAD) 之序列相同。從 1995 至 2002 年吾人從南台灣收集 22 株克沙奇 B5 病毒，其中四株來自於四例新發作第一型糖尿病病人，另收集已發表原型株 2 株。所有 22 株克沙奇 B5 病毒株皆以免疫螢光及中和試驗鑑定確認。吾人比較 22 株引致關第一型糖尿病和非引致糖尿病克沙奇 B5 病毒株其 2C 蛋白 300 個核酸序列，結果發現南台灣 22 株克沙奇 B5 病毒核酸序列僅有 0.3–10% 之相異性。四例第一型糖尿病病人克沙奇 B5 病毒核酸甘序列僅有 2.4–3.4% 之相異性。此南台灣 22 株克沙奇 B5 病毒株與已發表克沙奇 B5 病毒原型株 2C 蛋白核酸序列比較顯示有 18.4–24.1% 之差異。除了 96-1214 病毒株外，所有其他與糖尿病相關及非糖尿病之病毒株 2C 蛋白亦皆含有 PEVKEK。本研究顯示南台灣 22 株克沙奇 B5 病毒核酸序列有高度相似性，引致與非引致糖尿病之克沙奇 B5 病毒株應無不同，吾人認為第一型糖尿病之病因中其他因素，如 HLA 基因易致病性及自體免疫抗體可能亦扮演重要之角色。

關鍵詞：核酸序列定序，克沙奇 B5 病毒，第一型糖尿病

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高雄醫學大學醫學院實驗診斷學

高雄市 807 自由一路 100 號