Enterovirus RNA Is Found in Peripheral Blood Mononuclear Cells in a Majority of Type 1 Diabetic Children at Onset

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We have studied the occurrence of enterovirus (EV)-RNA at the onset of childhood type 1 diabetes in all 24 new cases of childhood type 1 diabetes during 1 year in Uppsala county, Sweden. We also studied 24 matched control subjects and 20 siblings of the patients. RNA was isolated from peripheral blood mononuclear cells and EV-RNA detected by RT-PCR. Primers (groups A and B) corresponding to conserved regions in the 5′ noncoding region (NCR) of EV were used in the PCRs, and the amplicons were sequenced. By the use of group A primers, EV-RNA was found in 12 (50%) of the 24 type 1 diabetic children, 5 (26%) of 19 siblings, and none of the control subjects. Both patients and siblings showed a higher frequency of EV-RNA compared with the control subjects. The group B primers detected EV-RNA in all three groups but did not show statistically significant differences between the groups. The EV-RNA positivity with the group B primers was 11 (46%) of 24 in the type 1 diabetic children, 11 (58%) of 19 in the siblings, and 7 (29%) of 24 in the control subjects. The significant difference between groups seen with the group A primers but not with the group B primers might indicate the existence of diabetogenic EV strains. The phylogenetic analysis of the PCR products revealed clustering of the sequences from patients and siblings into five major branches when the group A PCR primers were used. With the group B primers, the sequences from patients, siblings, and control subjects formed three major branches in the phylogenetic tree, where 6 of the 7 control subjects clustered together in a sub-branch of CBV-4/VD2921. Seven of the type 1 diabetic children clustered together in another sub-branch of CBV-4/VD2921. Five of the type 1 diabetic children formed a branch together with the CBV-4/E2 strain, four clustered together with CBV-5, and one formed a branch with echovirus serotype. The presence of EV-RNA in the blood cells of most newly diagnosed type 1 diabetic children supports the hypothesis that a viral infection acts as an exogenous factor. In addition, sequencing of the PCR amplicons from the type 1 diabetic children, their siblings, and matched control subjects might reveal differences related to diabetogenic properties of such a virus. *Diabetes* 51: 1964–1971, 2002

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CBV, Coxsackievirus B; CPE, cytopathic effect; EMEM, Eagle's minimum essential medium; EV, enterovirus; IAA, insulin autoantibody; ICA, islet cell antibody; NCR, noncoding region; NT, neutralizing antibody; PBMC, peripheral blood mononuclear cell; RT, reverse transcription.

Juvenile insulin-dependent diabetes (type 1 diabetes) is a common, severe chronic disease that is associated with significant secondary morbidity and that imposes a considerable burden on health care systems. The incidence of type 1 diabetes shows large geographic region–dependent variation, with Sweden and Finland having the highest figures. It is generally believed that both genetic and environmental factors play significant roles in the etiology of type 1 diabetes. Certain HLA-DQ and -DR haplotypes have been associated with increased risk of developing type 1 diabetes (1,2), whereas others provide some degree of protection (3). Hence, human genetics cannot explain the geographical differences in the incidence of the disease, and there is plenty of evidence suggesting a major role for environmental factors, such as virus infections, in this context. Enterovirus (EV) infection has for a long time been among the most plausible candidates for the necessary environmental component in the etiology. The genus *Enterovirus* within the family *Picornaviridae* consists of positive-stranded RNA viruses and comprises polioviruses, Coxsackieviruses A and B, echoviruses, and the numbered EVs 68–71. So far, a total of 64 immunologically distinct serotypes are known to cause infections in humans, ranging from asymptomatic to fatal.

Type 1 diabetes results from a progressive loss of functional insulin-producing β-cells in the Langerhans islets of the pancreas. An autoimmune response toward islet cell antigens can be demonstrated in prediabetic individuals, often several years before the onset of overt clinical disease. There is abundant literature on various aspects of humoral autoimmune responses (islet cell antibody [ICA], insulin autoantibody [IAA], GAD65, etc.), although it is usually held that the tissue damage would be caused by cytolytic autoimmune T-cells. GAD65, heat shock protein 60/65, proinsulin, and several other β-cell components have been suggested as key antigens because of successful modification of the natural course of disease in the NOD mouse, the autoimmune model of type 1 diabetes. However, there is no consensus about the identity of a putative "primary antigen" or about the mechanisms of onset of the autoimmune response in humans.

EV infections have for a long time been suspected of having a role as putative precipitating factors for the onset

TABLE 1
List of primers used in RT-PCR and sequencing in this study

Primer	Sequence	Polarity	Location*
E 1	5′ CACCGGATGGCCAATCA 3′	Antisense	640
ECBV1	5′ GGTACCTTTGTGCGCCTGTT 3′	Sense	65
ECBV2	5′ CAAGCACTTCTGTTTCCCCGG 3′	Sense	160
ECBV4	5′ ATTGTCACCATAAGCAGCCA 3′	Antisense	603
ECBV5	5′ GATGGCCAATCCAATAGCT 3′	Antisense	640

*Positions refer to the Coxsackievirus B4/E2 strain (GenBank accession no. S76772) and the B4/J.V.B strain (GenBank accession no. D00149)

of clinical type 1 diabetes. Epidemiological data showed an increased incidence of type 1 diabetes after epidemics caused by EVs (4). Coxsackievirus B4 (CBV-4) has been isolated from some patients with acute onset of type 1 diabetes, and some of these isolates have been shown to be able to induce a diabetes-like condition in mice (5). In cell cultures of murine (6) or human (7,8) islet cells, several CBV-4 strains are able to replicate, although the virus titers and the appearance of cytopathic effect (CPE) differs. Some of these strains suppress insulin release in response to high glucose in human islets, thus suggesting diabetogenic properties. Two studies revealed strains of CBV-4 that were able to establish a persistent infection in human islet cells (9,10), and in one of these, the infection was associated with α-interferon synthesis in the β-cells (10). Elevated levels of CBV-specific IgM-class antibodies, as a marker of recent or ongoing infection, have frequently been observed in recent-onset type 1 diabetic patients (11–13). More recent evidence of ongoing EV infection in newly diagnosed type 1 diabetic patients is the presence of EV-RNA in blood samples from these patients (14–16). EV infections appear to coincide with seroconversion to or enhancement of the immune response to islet cell antigens (ICA), insulin (IAA), or GAD65 (17,18). CBV shares a motif of six amino acids with GAD65 (19,20).

Studies also suggest that maternal EV and rubella virus infections during pregnancy will increase the risk of type 1 diabetes in the child (21–23). It has been shown that there may be multiple infections, with the development of ICAs leading to gradual β-cell destruction. Alternatively, the EV infection may persist after infection during fetal life because of the immature immune system, leading to the destruction of the β-cells by a direct or indirect mechanism.

The aims of this work were to detect EV-RNA in peripheral blood mononuclear cells (PBMCs) and to study the occurrence of EV infections in a 1-year cohort of recently diagnosed type 1 diabetic children and their matched control subjects. A further aim was to study the intrafamiliar spread of this infection by analyzing siblings of the type 1 diabetic children. Sequencing of the PCR amplicons from the 5′- noncoding region (NCR) of the viruses from type 1 diabetic children, their siblings, and matched control subjects might reveal differences related to diabetogenic properties of such a virus.

RESEARCH DESIGN AND METHODS

Children with type 1 diabetes, siblings, and control subjects. The study comprises all 24 children below the age of 16.0 years with newly diagnosed diabetes during 1 year in Uppsala county, which has slightly less than 300,000 inhabitants. Six of the type 1 diabetic children were female and 18 male, with a mean age of 8.4 years (range 1.6–15.7). They had a mean history of polydipsia and polyuria of 18 days, with a wide variation (1–60 days). Mean HbA_{1c} was 9.8% (range 6.5–16.5; upper reference limit 5.0), and the first blood glucose was between 15.5 and 40.7 mmol/l (mean 25.0). They were all in relatively good condition, all were conscious, and only three had a pH <7.30. The first blood was taken within 1 week in all cases, except in one boy who fell ill in another part of the country (day 26). The second blood sample was taken 2–6 months after the diagnosis. The control group consisted of 24 age- and sex-matched control subjects from the same county or neighboring counties, with the exception of two control subjects who were from the north of Sweden. The control subjects were recruited within 2 months after the proband, from among patients without evidence of ongoing infection. Samples were also taken from 20 siblings of the type 1 diabetic children who were willing to participate (mean age at the first sample 12.4 ± 6.0 years). The first blood sample from each sibling was obtained at or close (within 6 weeks) to the diagnosis of the index case. The study was approved by the ethics committee of the Medical Faculty at Uppsala University.

Viruses and cells. Three strains of CBV-4 were used, V89-4557 and VD2921 (two plaque-purified strains isolated from patients suffering from aseptic meningitis), and the E2-Yoon strain (shown to be diabetogenic in mice). All three strains are readily neutralized with a standard polyclonal neutralizing anti–CBV-4 serum (American *Type Culture* Collection). Two of the strains have been sequenced and compared phylogenetically (9,24), and they have earlier been used for the study of viral involvement in type 1 diabetes (5,7–9). Green monkey kidney (GMK) cells were used for the neutralization test. They were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% newborn bovine serum.

Measurements of neutralizing antibodies, antibodies against GAD65, and EV-IgM. For analyses of neutralizing antibodies, 5 μl of the serum specimens were serially diluted with EMEM in twofold steps from 1:20 to 1:2,560. The diluted sera and EMEM containing 100 of the 50% tissue culture infective dose ($TCID_{50}$) of the virus/0.1 ml were mixed and incubated for 90 min at 37°C. The mixtures were then transferred to GMK cells cultured in 96-well plates. The titers were recorded after 4–8 days. Antibodies against GAD65 in serum samples from the type 1 diabetic group, the siblings, and the control group were measured with Diamyd's Anti-GAD65 RIA (Mercodia, Uppsala, Sweden). Using a cutoff of 9.5 units/l, the specificity was 99% and the sensitivity was 74%. IgM antibodies against EV were measured at the Karolinska Institute, Stockholm (25).

RNA and DNA preparation. PBMCs were isolated from the type 1 diabetic children, their siblings, and healthy control subjects by centrifugation of blood samples with Lymphoprep (Nycomed Pharma, Oslo). They were stored at −70°C until the DNA/RNA extraction. Total RNA was extracted from PMBCs using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). The RNA was stored at −70°C. DNA from patients and control subjects was extracted from PMBCs using a QIAamp DNA Mini Blood Kit (QIAGEN).

HLA typing. HLA typing of DRB1 and DQB1 genes was achieved with Dynal Classic SSP (Branborough, U.K.), a PCR-based method using sequence-specific primers for each HLA allele and visualization of the PCR products on a 1.2% agarose gel. All of the type 1 diabetic children, 23 of the 24 control subjects, and 11 of the 12 EV-PCR–positive siblings were HLA typed.

Primers for EV-PCR. The primer pairs ECBV-1 to -5 correspond to highly conserved regions within the 5′-NCR of the enteroviral genome (Table 1). The external primers ECBV5 and ECBV1 generate a 600-bp fragment, whereas the use of an internal primer for PCR group A ECBV4 (downstream primer) and ECBV1 (upstream primer) generates a 539-bp PCR product. The external primers used in group B were ECBV4 and ECBV1. The internal primers for the PCR group B ECBV4 (downstream primer) and ECBV2 (upstream primer) generated a 436-bp PCR product.

Reverse transcription. The reverse transcription (RT) mixture (12 μl) containing 11 μl extracted RNA with 1 μl of negative-strand primer (E1) (Table 1) was heated for 10 min at 70°C and put on ice. Then, 7 μl of master mix containing 4 μl of 5 × first-strand buffer (250 mmol/l Tris-HCl, 375 mmol/l KCl, and 15 mmol/l $MgCl_2$), 2 μl of 0.1 mol/l dithiothreitol, and 1 μl of 10 mmol/l concentration of each dNTP (Life Technologies) was added, and the mixture was incubated for 2 min at 42°C. This was followed by the addition of

TABLE 2
EV-PCR results from PBMCs from type 1 diabetic children at onset and degree of homology to EV genotypes

Patient	Sex	Age (years)	RT-PCR Primer group A	RT-PCR Primer group B	EV genotype homology (identity)	EV-IgM	NT titer E2	NT titer V89-4557	NT titer VD2921	Anti-GAD65 (units)	HLA-DRB1*	HLA-DQB1*
P1	F	8	+	+	92.4 (CBV-5)	—	80	—	40	300	04, 08	04, 04
P2	M	7	−	+	92.3 (echo-V-5)	—	640	—†	>2,560	—	01, 13	05, 06
P3	M	11	+	+	92.5 (CBV-4/E2)	—	—	—	20	50	04, 11	03, 03
P4	M	4	+	−	92.3 (CBV-4)	200	160	—	20	—	03, 08	02, 04
P5	M	13	+	−	91.5 (CBV-4/VD2921)	—	—	—	—	90	03, 04	02, 03
P6	M	8	+	−	92.8 (CBV-4/E2)	200	—	160	—	180	04, 13	03, 04
P7	M	7	−	+	93.4 (CBV-4/E2)	—	320	—	160	250	03, 09	02, 03
P8	M	13	−	−		—	320	—	—	—	04, 04	03, 03
P9	M	13	+	−	99.8 (CBV-5)	—	—	40†	20	—	03, 01	02, 05
P10	M	4	+	−	98.2 (CBV-5)	—	—	—	20	—	04, 07	02, 03
P11	M	8	−	−		ND	20	20	—	100	04, 13	06, 06
P12	M	4	+	−	96.6 (CBV-4/VD2921)	200	20	—	—	—	01, 04	05, 03
P13	F	9	−	+	95.4 (CBV-4/VD2921)	—	40	—†	—	13	04, 11	05, 05
P14	M	3	+	+	97.0 (CBV-4/VD2921)	—	—	20	20	100	03, 14	06, 06
P15	M	11	+	−	96.8 (CBV-4/VD2921)	—	20	20	—	—	01, 03	02, 05
P16	F	14	−	+	93.6 (CBV-4/E2)	—	160	—	20	250	01, 04	03, 05
P17	F	6	−	−		—	20	—	—	11	03, 13	02, 06
P18	M	1	+	+	97.8 (CBV-4/VD2921)	—	—	—	—	40	03, 04	02, 02
P19	M	16	−	−		ND	—	—	40	190	03, 04	02, 02
P20	M	8	−	−		ND	80	320	160	26	03, 04	02, 03
P21	F	6	−	+	93.6 (CBV-4/E2)	ND	—	80†	160	—	03, 04	02, 03
P22	F	10	+	+	98.2 (CBV-4/VD2921)	—	80	80†	40	10	04, 04	03, 03
P23	M	10	−	−		—	—	20†	40	24	01, 04	03, 05
P24	M	13	−	+	98.7 (CBV-5)	200	160	80	20	12	01, 04	03, 05

ND, not done. †Significant rise in NT titer. Measurements of antibodies against EV (EV-IgM), neutralization antibodies against the CBV-4 strains E2-Yoon, V89-4557, and VD2921; and antibodies against GAD65 in serum from type 1 diabetic children at onset are shown. HLA-typing of the type 1 diabetic children was performed.

200 units of Superscript II (Life Technologies) and incubation for 60 min at 42°C. The reverse-transcribed samples were then denatured for 15 min at 70°C before storage on ice.

PCR. The PCR was performed in two steps. First, a PCR was carried out by using the two primer pairs ECBV5/ECBV1 (group A) and ECBV4/ECBV1 (group B). cDNA (2 μl) was amplified in a volume of 50 μl containing 50 mmol/l KCl, 20 mmol/l Tris-HCl (pH 8.4), 2.5 mmol/l $MgCl_2$, 0.1 mg/ml BSA, 0.2 mmol/l of the dNTPs, 50 ng of each primer, and 2 units of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ) in a DNA thermal cycler-Touchgen (Techne, Cambridge, U.K.) with the following profile: 94°C for 4 min, followed by 40 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 1 min, followed by a final extension step at 72°C for 5 min. A second-run PCR was performed using primer pairs ECBV4/ECBV1 in group A (ECBV5/ECBV1) and ECBV4/ECBV2 in group B (ECBV4/ECBV1). The PCR was performed as described above. Briefly, 2 μl of the amplified products were added to the PCR mixture, consisting of 10 μl of 10 × PCR buffer, 4 units *Taq* DNA polymerase, and 100 ng each of primers ECBV-1 to -4. The mixture was adjusted to a volume of 100 μl with water. The PCR was performed as in the first PCR, but using 50°C instead of 45°C for the annealing temperature. Precautions were taken to avoid any kind of contamination with extraneous nucleic acid. A negative control (no RNA) was used in each PCR. RT and the first and second PCRs were performed in separate locations in the laboratory.

Sequence analysis. PCR products from the second PCR were identified by 1.5% agarose gel. The positive PCR products with correct size (539 bp with the primers ECBV4/ECBV1 and 436 bp with the primers ECBV4/ECBV2) were purified using QIAquick PCR Purification Kit (QIAGEN). Both strands of the DNA fragments were sequenced using an automatic ABI Prism 310 sequencer by the Big Dye–labeled terminator method using Amplitaq DNA polymerase FS (Perkin-Elmer). The sequencing primers were the same as those used in the second run of PCR amplification (ECBV4, ECBV1, and ECBV2).

To estimate genetic relationship, the nucleotide sequences from EV-positive siblings of patients, from control subjects, and from known sequences of other EV serotypes (deposited in GenBank) were aligned using the CLUSTAL W (1.5) multiple sequence alignment program (26). The analyses were performed using the programs DNADIST and NEIGHBOR-joining included in the PHYLIP package (PHYLIP Phylogeny Inference Package, Version 3.5p; Department of Genetics, University of Washington, Seattle, WA) (27). To check the reliability of the branches defined by the phylogenetic tree, we used SEQBOOT (100 replicates), which was also included in the PHYLIP package. Boot-strapping of 1,000 replicates was performed.

Statistical analysis. Statistical analyses of four field tables were performed using Fisher's exact two-tailed test. A P value <0.05 was considered to indicate a statistical significance. The statistical package SPSS for Windows, Version 10.0, was used in statistical analyses.

RESULTS

As shown in Table 2, EV-RNA was found in PBMCs from 12 (50%) of the 24 newly diagnosed type 1 diabetic patients and 5 (26%) of 19 siblings, but in none of the control subjects (Table 3) with the use of primers ECBV4/ECBV1 (group A). With the use of primers ECBV4/ECBV2 (group B), EV-RNA positivity was detected in PBMCs from 11 (46%) of the 24 type 1 diabetic children, 11 (58%) of 19 siblings, and 7 (29%) of the 24 control subjects (Tables 2 and 3). One of the EV-PCR–positive siblings (P20S) belonged to an EV-PCR–negative family. The total number of EV-RNA–positive patients was 18 (75%), among the siblings the corresponding figure was 12 (63%) of 19, and in the control group the figure was 7 (29%) of 24. With the use of group A primers, 50% of the type 1 diabetic children revealed EV-PCR positivity, compared with none (0%) of the 24 control subjects, a statistically significant difference ($P < 0.001$). The difference in group A EV-PCR positivity between the siblings (5 of 19) and the type 1 diabetic children (12 of 24) was not significant, but siblings had a higher frequency of EV-PCR positivity compared with the control subjects ($P < 0.05$).

Two phylogenetic trees were constructed by comparing the sequences from EV-PCR amplicons obtained from type 1 diabetic children, siblings, and control subjects and

TABLE 3
EV-PCR results from EV-positive siblings and control children and the degree of homology to EV genotypes

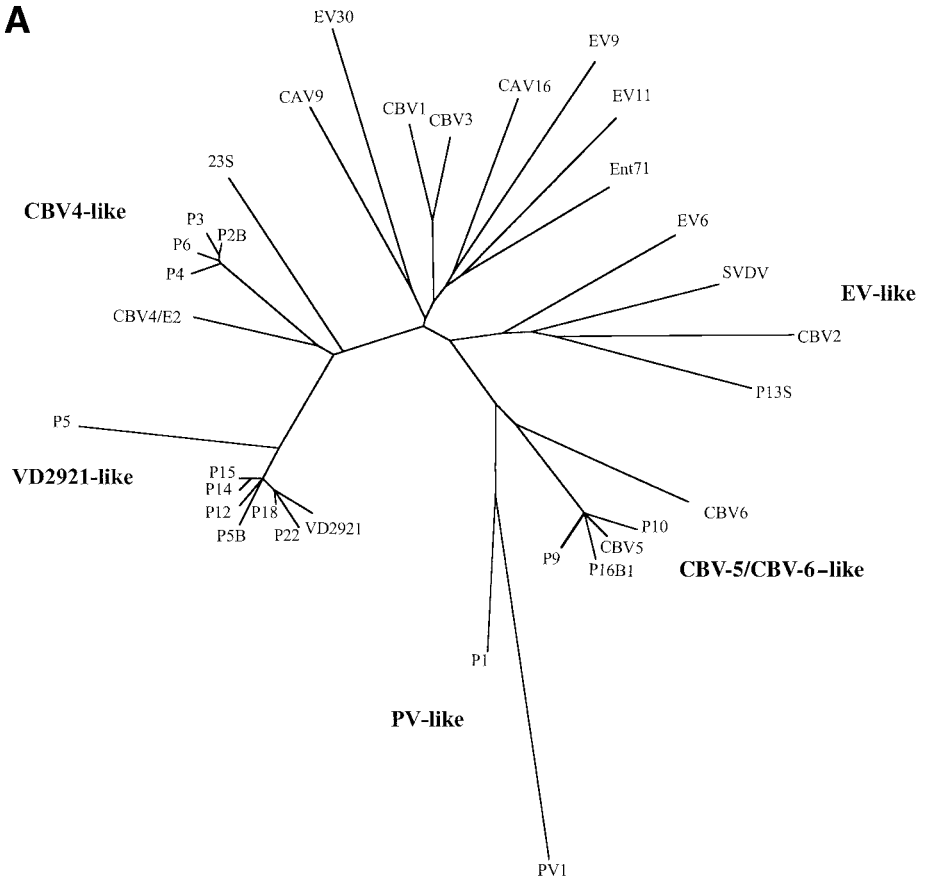
Sibling	Sex	Age (years)	RT-PCR Primer group A	RT-PCR Primer group B	EV genotype homology	EV-IgM	NT titer E2	NT titer V89-4557	NT titer VD2921	Anti-GAD65 (units/l)	HLA-DRB1*	HLA-DQB1
P1S	F	8	−	−		ND	320	—†	20	50	03, X	02, 02
P2B	M	9	+	−	92.7 (CBV-4/E2)	ND	640†	160	320	—	07, 11	02, 02
P5B	M	26	+	+	96.6 (CBV-4/VD2921)	ND	1,280	—	160	—	04, 14	03, 03
P7S	F	5	−	+	93.9 (CBV-4/E2)	ND	320	40	160	—	03, 07	02, 02
P9S	F	18	−	+	97.7 (CBV-4/VD2921)	ND	—	—	20	—	ND	ND
P10B	M	6	−	+	92.9 (CBV-4/E2)	ND	—	—	—	—	07, 09	02, 03
P13S	F	7	+	+	87.9 (SVDV)	ND	80	160	2,560	—	11, X	ND
P16B1	M	11	+	+	99.8 (CBV-5)	ND	160	—	—	80	01, 04	05, 03
P16B2	M	10	−	+	93.9 (CBV-4/E2)	ND	160	20	—	—	01, 13	05, 06
P20S	F	7	−	+	93.6 (CBV-4/E2)	ND	160†	160	160	—	13, X	06, 06
P21S	F	10	−	+	93.1 (CBV-4/E2)	ND	—	320	20	—	03, 04	02, 03
P22B	M	13	−	+	90.5 (CBV-4/VD2921)	ND	>1,280	320	20	—	01, 04	05, 03
P23S	F	9	+	+	89.2 (CBV-4/E2)	ND	—	160	40	—	ND	ND
C9	M	13	−	+	ND	ND	—	—	—	—	04, 13	06, 03
C18	M	1	−	+	ND	ND	—	—	20	—	ND	ND
C19	M	16	−	+	ND	ND	80	20	20	10	15, 13	06, 06
C20	M	8	−	+	ND	ND	—	160	80	—	16, 03	02,
C21	M	6	−	+	ND	ND	320	80	40	—	04, 08	03, 04
C22	M	10	−	+	ND	ND	1,280	—	—	—	04, 15	03, 06
C23	M	10	−	+	ND	ND	40	80	40	—	07, 15	03, 06

Measurements of neutralizing antibodies against the CBV-4 strains E2-Yoon, V89-4557, and VD2921 and antibodies against GAD65 in serum from siblings and control children are shown. HLA typing of the siblings and control subjects was performed. ND, not done; P, patient; B, brother; S, sister; C, control. †Significant rise in NT titer. X = any HLA-DRB1 allele except *04. The figure for EV genotype homology belongs to primer group A if it is positive; otherwise it belongs to group B.

previously published EVs (Fig. 1*A* and *B*). Figure 1*A* shows the phylogenetic analysis of the 5′-NCR sequences obtained with the ECBV4/ECBV1 primers (group A), which revealed clustering of the sequences into five major branches. CBV-1, CBV-3, and the other EV formed their own cluster (bootstrap 99%). Six of the patients and one of the type 1 diabetic children's siblings (subject P5B) clustered with the CBV-4 strain VD2921 (VD2921-like), and one patient (subject P5) formed a sub-branch in the VD2921-like cluster. P2B, P23S, P3, P4, and P6 were found in the same cluster as the CBV-4/E2 (CBV-4–like). The sequence from P13S was clustered with CBV-2, echovirus-6, and SVDV (EV-like). P9, P10, and P16B1 sequences were more closely related to CBV-5 and -6 (CBV-5/CBV-6–like, respectively). The sequence of one patient (subject P1) formed a separate cluster with poliovirus, although this patient was clearly in the same branch as P9, P10, and P16B1 (PV-like). The sequence alignment showed that the nucleotide sequence of patient 1 was 79.1% similar to poliovirus type 1. The CBV-4/E2–like, CBV-4/VD2921–like, and CBV-5/CBV-6–like groups were strongly supported by bootstrap values of 94–100%, whereas the bootstrap score for the echovirus-like group was 61%.

Figure 1*A* shows the phylogenetic analyses of the 5′-NCR sequences obtained with the group B primers. They segregated into three major clusters: CBV-5/CBV-6-like, echovirus-like, and CBV-4/E2–like. In this tree, unlike the tree obtained with the group A primers, VD2921 was clustered together with CBV-4/E2. Eight positive amplifications (subjects P7, P16, P21, P7S, P10B, P16B2, P20S, and P21S) were closely grouped together in one of the sub-branches formed with the CBV-4/E2 strain and VD2921 with 100% bootstrap support. Subjects P13, P9S, and P22B were clustered with VD2921 with 95.4–97.7% nucleotide identity. Six of the control children formed the other sub-branch of the CBV-4/E2–like cluster. One patient and one control subject (subjects P24 and C9) clustered with CBV-5/CBV-6–like, and the sequence of one patient (subject P2) was related to echovirus (EV-like cluster). Comparison of these two trees revealed some variation in the branching order of some subgroups, some variation in bootstrap values for certain nodes, and also a variation in branch lengths. In addition, the branching order of the CBV-5/CBV-6–like and echovirus-like clusters differed between the two trees.

IgM antibodies against EV were found in 4 of 24 patients (subjects P4, P6, P12, and P24). All of these were EV-PCR–positive. Two of the EV-IgM–positive patients (subjects P6 and P24) also revealed antibodies against GAD65. No analyses of EV-IgM antibodies were performed in serum samples from siblings or control subjects. The neutralizing antibody (NT) titers against CBV-4 differed somewhat, depending on which strain was used in the tests (Tables 2–4). The NT titer in sera from one patient could be negative against one of the CBV-4 strains and as high as 2,560 against one of the other strains. The number of NT-positive samples against the CBV-4 strains did not vary significantly among the patients, siblings, and control subjects. No significant difference in titer levels against the three CBV-4 strains was obtained when the three groups were compared. In 15 of the patients (subjects P1–3, P5, P8–10, P13, P16, P17, and P19–23), a convalescent serum sample was taken 2–3 months after the onset of type 1 diabetes and, as shown in Table 5, 7 of the type 1 diabetic children (subjects P2, P5, P9, P13, and P21–23) had a significant rise in NT titer between the acute and convalescent sera against V89-4557. In total, 7 (41%) of 17 revealed a significant rise in NT titer, and 1 of these children was EV-PCR–negative (subject P23). Five of the



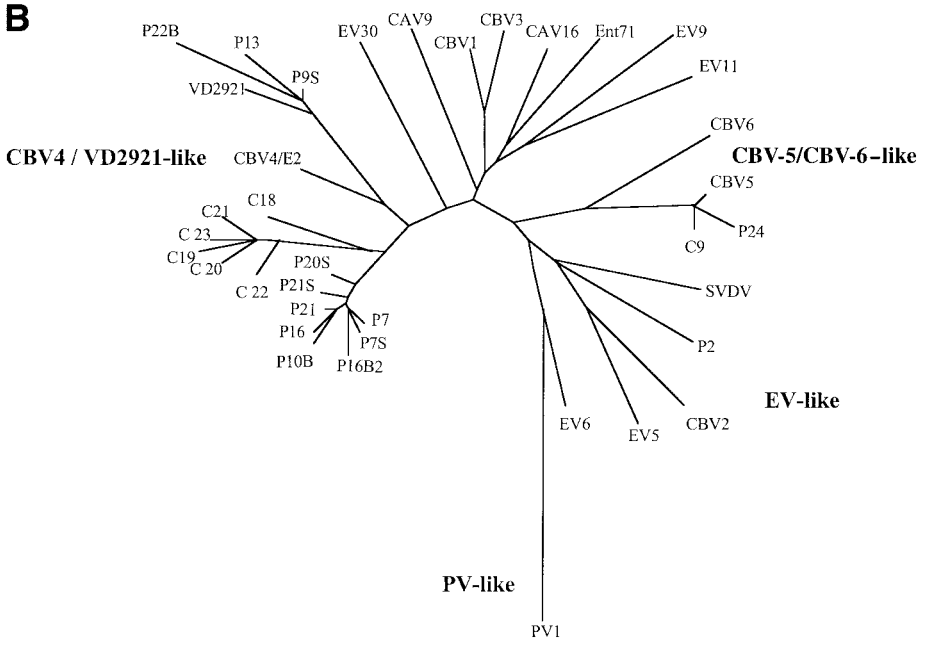


FIG. 1. Phylogenetic analysis performed of EV sequences from type 1 diabetic patients, siblings, control subjects, and human EVs using the neighbor-joining method. Phylogenetic trees depicting genetic relationships between human EVs and amplicons from type 1 diabetic patients detected by RT-PCR are given for group A (*A*) and group B (*B*). Trees were based on the sequence alignment of the 496 nucleotides (group A) and 394 nucleotides (group B) from 5′-NCR. EV sequences used in this analysis were CBV-4 strain VD2921 (9); CBV-1 (accession no. M16560); CBV-2 Ohio-1 reference strain (accession no. AF081485); CBV-3 (accession no. M16572); CBV-4 (accession no. S76772); CBV-5 (accession no. AF114383); CBV-6 (accession no. AF225478); CAV-9 (accession no. D00627); CAV-16 (accession no. NC001612); echovirus 5 (echo-V-5; accession no. AF083069); echo-V-6 (accession no. NC001657); echo-V-9 (accession no. X84981); echo-V-11 (accession no. X80059); echo-V-30 (accession no. NC000873); EV-71 (accession no. NC001769); SVDV (accession no. X54521); and poliovirus type 1 (PV-1; accession no. V01149).

type 1 diabetic children were too young (age ≤5 years) for a second serum sample to be obtained (they were all EV-PCR–positive), and we were not able to get a second sample from three of the patients for other reasons. Among the siblings, three revealed significant NT titer rises against CBV-4/CBV-2 against the E2-Yoon strain and one revealed a rise against the V89-4557 strain. All but one of these siblings (subject P1S) were EV-PCR–positive. In total, 10 children revealed significant rises in NT titer against CBV-4, and in 8 of them, the finding could be confirmed by a positive EV-PCR. Comparison of sequencing results with the presence of NT titer against the different CBV-4 strains revealed that of the 13 patients with CBV-4–like sequences, 2 showed a significant rise in NT titer against the

TABLE 4
Neutralization antibody titers against three strains of CBV-4 in acute sera from type 1 diabetic children, their siblings, and control subjects

	VD2921		E2-Yoon		V89-4557	
Group	Titer*	Positive†	Titer	Positive	Titer	Positive
Type 1 diabetic patients	224	15 of 24 (62.5)	151	14 of 24 (58)	84	10 of 24 (42)
Siblings	274	23 of 20 (65)	377	13 of 20 (65)	174	10 of 20 (50)
Control subjects	49	13 of 24 (54)	243	11 of 24 (46)	125	11 of 24 (46)

*Mean NT titer; †number of NT titer–positive. Data for positive titers are *n* (%).

V89-4557 strain and 1 had a rise in titer against the E2-Yoon strain (Table 2). We could not obtain convalescent serum samples from five of these patients. In the remaining five type 1 diabetic children, no confirmation of the observed sequence homology with CBV-4 could be obtained with the use of NT; however, some of these patients showed high NT titers against one or more of the CBV-4 strains already in the acute serum. No NT tests were performed against other serotypes of CBV or against other EVs.

Antibodies against GAD65 were found in 16 (67%) of the 24 acute sera from the type 1 diabetic children, compared with in 2 (10%) of the 20 siblings ($P < 0.01$) and 1 (4%) of the 23 control subjects ($P < 0.001$) (Tables 2 and 3). These antibodies were also detected in 13 of the 17 convalescent sera (subjects P1, P3, P5, P7, P8, P10, P11, P13, P16, P17, P19, P21, and P23). In total, 18 (75%) of the 24 type 1 diabetic children revealed these antibodies at the onset or 2 months later. Among the siblings, 2 (10%) of 20 had these antibodies in the acute sera, and both of these subjects were negative when the convalescent sera (taken 2–3 months later) were analyzed. One of the control subjects (subject C19) had a low level of antibodies against this autoantigen. Of the two anti-GAD65–positive siblings, one was EV-PCR–positive and one had a significant rise in NT titer against V89-4557. Group B–positive individuals (type 1 diabetic children, siblings, and control subjects) more often than group B–negative individuals revealed antibodies to GAD65 ($P < 0.05$). Group A positivity, however, was not significantly associated with antibodies against this autoantigen. Individuals positive in both group A and group B EV-PCR also had a higher frequency of antibodies against GAD65 than individuals positive in just group A or group B or those who were EV-PCR–negative ($P < 0.001$).

HLA-DRB1 and -DQB1 alleles for each patient are presented together with EV-PCR results in Table 2. The allele HLA-DRB1*04, which has been linked to increased risk for type 1 diabetes in earlier studies, was overrepresented in the type 1 diabetic group, being present 71% of the type 1 diabetic children compared with 30% of the control subjects ($P < 0.01$), whereas the alleles DR and HLA-DQB1*06 were more common among control subjects. None of the type 1 diabetic children, compared with 39% of the control subjects, carried the DRB1*15 allele ($P < 0.01$), and 17% of the patients, compared with 70% of the control subjects, carried the DQB1*06 ($P < 0.01$) allele.

DISCUSSION

EV-PCR positivity among newly diagnosed type 1 diabetic children and adults has been shown before (14–16), but to our knowledge, this is the first time that primers that detect EV that might be diabetogenic have been used. In the earlier studies, the frequencies of EV-PCR positivity varied (27, 67, and 37%, respectively) and, compared with the total EV-PCR positivity shown in this study (75%), they were all lower. Chehadeh et al. (15) also detected EV-RNA in patients previously diagnosed with type 1 diabetes (both children and adults), indicating a persistent infection or an increased susceptibility in this group of patients for EV infections. A possible explanation for the high frequency of EV-RNA positivity in our study might be that RNA was extracted from peripheral blood cells instead of from plasma. It has been reported that peripheral blood cells can harbor EVs during and after a viremic phase (28). During viremia, EVs are transported both intracellularly by mononuclear cells (lymphocytes or monocytes) and extra-

TABLE 5
Significant rises in neutralization antibody titers (bold) and titers against GAD65 in samples from type 1 diabetic children and in samples from siblings obtained at onset and 2 months later

		NT titers							
		V89-4557		E2-Yoon		VD2921		Anti-GAD65	
Patient	Sex	A	C	A	C	A	C	A	C
P2	M	—	**640**	640	640	>2,560	>2,560	—	—
P5	M	—	**80**	—	—	—	—	90	300
P9	M	**40**	**160**	—	—	160	80	—	—
P13	F	—	**320**	40	80	—	—	13	400
P21	M	**40**	**160**	—	—	160	80	—	10
P22	F	**80**	**320**	80	20	40	20	10	10
P23	M	**20**	**320**	—	—	—	20	24	23
P1S	F	—	**160**	320	160	20	40	50	—
P2B	M	160	160	**640**	**5,120**	320	320	—	—
P20S	F	80	40	**160**	**2,560**	80	80	—	—

A, acute sera; C, convalescent sera.

cellularly in serum, indicating better chances to detect viral RNA in the PBMC fraction compared with serum. Another possible explanation could be the selection of primers used in this study. As shown in the results, different pairs of primers (groups A and B) resulted in different frequencies of EV-RNA positivity among the type 1 diabetic children, their siblings, and the control subjects. It is noteworthy that by the use of the group A primers, 12 of the type 1 diabetic children revealed EV-PCR positivity, compared with none of the control subjects ($P < 0.001$) and only five of the siblings. This difference in detection might indicate important differences related to diabetogenicity in the nucleotide sequences of the specific annealing sites of the primers. Other differences in this study compared with those above (14–16) are that samples from the siblings of the type 1 diabetic children were also analyzed and that we were able to compare the viral sequences from the three groups of children with each other and with the sequence of a strain of CBV-4 (VD2921) that is able to establish persistent infection in human islet cells (9).

Phylogenetic trees revealed that the amplicons from the infected siblings shared a high degree of homology with that of the respective type 1 diabetic children. Two of the branches of the tree obtained with the group A primers consist of E2- or VD2921-like sequences. Both of these CBV-4 strains have been shown to be "diabetogenic" (5,8,9,23). The similarity with CBV-5 and swine vesicular disease virus (SVDV) are new findings, although it has recently been shown that SVDV utilizes the same receptor as CBV (29), indicating that the tropism of these viruses is similar. The phylogenetic tree of group B, as in the tree obtained with the group A primers, revealed that the EV sequences in these type 1 diabetic children and their siblings also shared a high degree of homology. An interesting finding was that six of seven control subjects were clustered on a separate sub-branch of CBV-4, compared with the sequences of the type 1 diabetic children and their siblings. In addition, with the use of these primers, the VD2921 and the CBV-4/E2 strain had branches that clustered together. The high degree of similarity between the sequences from the type 1 diabetic children and their brothers or sisters indicates that both were infected with the same or a closely related strain of a serotype of EV.

Because the 5′-NCR is very conserved among all serotypes of EVs, sequencing of this part of the viral genome means that comparisons of the sequences only vaguely implicate a specific serotype. The most variable regions of the EV genome are within the genes coding for the capsid proteins VP1, VP2, and VP3, which are partially exposed on the virus surface. VP1 codes for the major antigenic sites and most type-specific neutralization determinants (30). Molecular typing of EVs should ideally focus on regions encoding determinants for neutralization. Mapping with monoclonal neutralizing antibodies has shown that, in particular, the NH_2-terminal part of VP1, encompassing the B–C loop, is a major antigenic region. It has been shown that amplification of the VP1-P2C region, using a single primer pair, enabled serotyping of >90% of the EVs (31,32). This has yet to be done with the samples in this study, but such an approach would make it possible to determine which serotype caused the infection and, possibly, the type 1 diabetes.

In line with the idea that VP1 is the major antigenic region, differences between NT titers obtained with the VD2921 strain and the "diabetogenic" E2 strain might be explained by differences in amino acid sequences in this region. Sequencing of the VD2921 strain has revealed the substitution of 11 amino acids in the structural protein VP1, near the fivefold axis, compared with the E2 strain (9). In this study all but two of the significant rises in NT titer were obtained against the V89-4557 strain. It has been shown earlier that the highest frequencies of IgM antibodies among newly diagnosed type 1 diabetic children were obtained when this strain was used as an antigen (13). This might indicate that infection with strains that have a high degree of homology with the VP1 region of this strain or related strains is more common among children at the onset of type 1 diabetes. Antibodies able to neutralize a virus are considered to be of high affinity, and neutralizing antibodies produced after EV infections are usually type-specific. However, if the child has the immunological memory of an EV infection from the past and is then infected by another EV, neutralizing antibodies to the first infection could be produced together with such antibodies against the second infection (33). This might also explain possible rises in NT titers against one EV and sequence homologies to another.

EV infections have been reported to be temporally associated with the appearance of islet-specific autoantibodies (17). The frequencies of antibodies against GAD65 obtained in this study are in line with results obtained by other authors (18). The finding in this study that EV-PCR positivity obtained with the group A primers alone was not associated with the presence of antibodies against GAD65 is not in line with other groups results (17). However, EV positivity with both groups of primers (A and B) was positively associated with GAD65 antibodies, which is in line with the findings of others and might also indicate that multiple EV infections are needed to induce antibodies to autoantigens (34). The mechanism by which EV infections may induce diabetes in humans is unknown (35–39); these viruses are known to induce pancreatitis, especially the CBVs, and it has also been shown that these viruses can infect human β-cells in vitro (7–10).

If the role of EV infections in the etiology of type 1 diabetes holds true, it will be important to resolve the diabetogenic mechanism of the virus. If the virus itself causes the cell destruction by direct cell lysis, a putative EV vaccine might prevent some of the cases of type 1 diabetes. In contrast, if immunological cross–reactions or exposure to ICAs, such as GAD65 or heat shock protein 60, causes inflammation, by the molecular mimicry hypothesis, immune responses induced by EV vaccines might also facilitate autoimmune processes in type 1 diabetes. Further studies should investigate whether single, multiple, or persistent infections are needed for type 1 diabetes development, or whether people with autoimmunity are more prone to EV infections. The PCR technology used in this study holds further promise of enabling researchers to finally type the candidate diabetogenic strains amplified from newly diagnosed type 1 diabetic patients.

In conclusion, EV-RNA was detected in the PBMCs of a majority of newly diagnosed type 1 diabetic children, and the sequences of the PCR amplicons from these children

revealed homology with CBV as well as other EVs. These results support the involvement of EV infections in the development of type 1 diabetes.

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REFERENCES

1. Kockum I, Sanjeevi CB, Eastman S, Landin-Olsson M Dahlqvist G, Lernmark Å: Complex interaction between HLA DR and DQ in conferring risk for childhood type 1 diabetes. *Eur J Immunogenet* 26:361–372, 1999
2. Undlien DE, Kockum I, Ronningen KS, Lowe R, Sanjeevi CB, Graham J, Lie BA, Akelsen HE, Lernmark Å, Thorsby E: HLA association in type I diabetes among patients not carrying high-risk DR3-DQ2 or DR4-DQ8 haplotypes. *Tissue Antigens* 54:543–551, 1999
3. Sanjeevi CB, Höök P, Landin-Olsson M, Kockum I, Kahlqvist G, Lybrand TP, Lernmark Å: DR4 subtypes and their molecular properties in a population-based study of Swedish childhood diabetes. *Tissue Antigens* 47:275–283, 1996
4. Fohlman J, Friman G: Is juvenile diabetes a viral disease? *Ann Med* 25:437–445, 1993
5. See DM, Tilles JG: The pathogenesis of viral-induced diabetes. *J Infect Dis* 171:1135–1138, 1995
6. Frisk G, Grapengiesser E, Diderholm H: Impaired Ca^{2+} response in mouse β-cells infected with coxsackie B or Echo virus. *Virus Research* 33:229–240. 1994
7. Roivainen M, Rasilainen S, Ylipaasto P, Nissinen R, Ustinov J, Bouwens L, Eizirik DL, Hovi T: Mechanisms of coxsackievirus-induced damage to human pancreatic beta-cells. *J Clin Endocrino Metab* 85:432–440, 1999
8. Frisk G, Diderholm H: Tissue culture of isolated human pancreatic islets infected with different strains of coxsackievirus B4: assessment of virus replication and effects on islet morphology and insulin release. *Int J Exp Diabetes Res* 1:165–175, 2000
9. Yin H, Berg A-K, Westman J, Hellerström C, Frisk G: Nucleotide sequence of a CBV-4 strain able to establish a persistent infection in human pancreatic islet cells: effects on insulin release, pro-insulin synthesis and cell morphology. *J Med Virol*. In press
10. Chehadeh W, Kerr-Conte J, Pattou F, Alm G, Lefebvre J, Wattre P, Hober D: Persistent infection of human pancreatic islets by coxsackievirus B is associated with alpha interferon synthesis in beta cells. *J Virol* 74:10153–10164, 2000
11. King M, Shaikh A, Bidwell D, Voller A, Banatvala J: Coxsackie-B-specific IgM responses in children with insulin-dependent (juvenile-onset; type 1) diabetes mellitus. *Lancet* 1:1937–1929, 1985
12. Frisk G, Friman G, Tuvemo T, Fohlman J, Diderholm H: Coxsackie B virus IgM in children at onset of type 1 (insulin-dependent) diabetes mellitus: evidence for IgM induction by a recent or current infection. *Diabetologia* 35:249–253, 1992
13. Frisk G, Diderholm H: Antibody responses to different strains of coxsackie B4 virus in patients with newly diagnosed type I diabetes mellitus or aseptic meningitis. *J Infection* 34:205–210, 1997
14. Nairn C, Galbraith DN, Taylor KW, Clements GB: Enterovirus variants in the serum of children at the onset of Type 1 diabetes mellitus. *Diabet Med* 16:509–513, 1999
15. Chehadeh W, Weill J, Vantyghem MC, Alm G, Lefebvre J, Wattre P, Hober D: Increased level of interferon-alpha in blood of patients with insulin-dependent diabetes mellitus: relationship with coxsackievirus B infection. *Infect Dis* 180:1929–1939, 2000
16. Andreoletti L, Hober D, Hober-Vandenberghe C, Fajardy I, Belaich S, Lambert V, Vantyghem MC, Lefebvre J, Wattre P: Coxsackie B virus infection and beta cell autoantibodies in newly diagnosed IDDM adult patients. *Clin Diagn Virol* 9:125–133, 1998
17. Hiltunen M, Hyöty H, Knip M: Islet cell antibody seroconversion in children is temporally associated with enterovirus infections. *J Infect Dis* 175:554–560, 1997
18. Kockum I, Lernmark Å, Dahlqvist G, Falorni A, Hagopian WA, Landin-Olsson M, Li LC, Luthman H, Palmer JP, Sanjeevi CB, Sundqvist G, Östman J: Genetic and immunological findings in patients with newly diagnosed insulin-dependent diabetes mellitus. *Horm Metab Res* 28:344–347, 1996
19. Lönnrot M, Hyöty H, Knip M, Roivainen M, Kumala P, Leinikki P, Åkerblom HK: Antibody cross-reactivity induced by the homlogous region in glutamic acid decarboxylase (GAD65) and 2C protein of coxsackievirus B4: Childhood Diabetes in Finland Study Group. *Clin Exp Immun* 104:389–405, 1996
20. Härkonen T, Puolakkainen M, Sarvas M, Airaksinen U, Hovi T, Roivainen M: Picornavirus proteins share antigenic determinants with heat shock proteins 60/65. *J Med Virol* 62:383–391, 2000
21. Dahlqvist CG, Frisk G, Ivarsson SA, Svanberg L, Forsgren M, Diderholm H: Indications that maternal coxsackie B virus infection during pregnancy is a risk factor for childhood-onset IDDM. *Diabetologia* 38:1371–1373, 1995
22. Otonkoski T, Roivainen M, Vaarala O, Dinesen B, Leipala JA, Hovi T, Knip M: Neonatal Type I diabetes associated with maternal echovirus 6 infection: a case report. *Diabetologia* 43:1235–1238, 2000
23. Menser MA, Forrest JM, Bransby RD: Rubella infection and diabetes mellitus. *Lancet* 14:57–60, 1978
24. Kang Y, Chatterjee NK, Nodwell MJ, Yoon JW: Complete nucleotide sequence of a strain of coxsackie B4 virus of human origin that induces diabetes in mice and its comparison with non-diabetogenic coxsackie B4 JBV strain. *J Med Virol* 44:353–361, 1994
25. Glimåker M, Samuelson A, Magnius L, Ehrnst A, Olcen P, Forsgren M: Early diagnosis of enteroviral meningitis by detection of specific IgM antibodies with a solid-phase reverse immunosorbent test (SPRIST) and μ-capture EIA. *J Med Virol* 36:193–201. 1992
26. Thompson JD, Higgins DG, Gibson TJ: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680, 1994
27. Felsenstein J: *PHYLIP: Phylogeny inference Package. Version 3:5p*. Seattle, WA, Department of Genetics, University of Washington, 1993
28. Vuorinen T, Vainionpää, Kettin H, Hyypiä T: Coxsackievirus B3 infection in human leucocytes and lymphoid cell lines. *Blood* 84:823–829, 1994
29. Maritino TA, Petric M, Weingartl H, Bergelson JM, Opavsky MA, Richardson CD, Modlin JF, Finberg RW, Kain KC, Willis N, Gauntt CJ, Liu PP: The coxsackie-adenovirus receptor (CAR) is used by reference strains and clinical isolates representing all six serotypes of coxsackievirus group B and by swine vesicular disease virus. *Virology* 25:271:99–108, 2000
30. Schmidth NJ, Dennis J, Lennette EH, Ho HH, Shnomoto TT: Antibody responses within the coxsackievirus group. *J Immunol* 95:54–69, 1965
31. Hyypiä T, Hovi T, Knowless NJ, Stanway G: Classification of enteroviruses based on molecular and biological properties. *J Gen Virol* 78:1–11, 1997
32. Caro V, Guillot S, Delpeyroux F, Crainic R: Molecular strategy for serotyping of human enteroviruses. *J Gen Virology* 82:79–91, 2001
33. Sadeharju K, Lönnrot M, Kimpimaki T, Savola K, Erkkila S, Kalliokoski T, Savolainen P, Koskela P, Ilonen J, Simell O, Knip M, Hyoty H: Enterovirus antibody levels during the first two years of life in prediabetic autoantibody-positive children. *Diabetologia* 44:818–823, 2001
34. Lönnrot M, Korpela K, Knip M, Ilonen J, Simell O, Korhonen S, Savola K, Muona P, Simell T, Koskela P, Hyoty H: Enterovirus infection as a risk factor for beta-cell autoimmunity in a prospectively observed birth cohort: the Finnish Diabetes Prediction and Prevention Study. *Clin Infect Dis* 31:1025–1031, 2000
35. Vreugdenhil GR, Schloot NC, Hoorens A, Rongen C, Pipeleers DG, Melchers WJ, Roep BO, Galama JM: Acute onset of type I diabetes mellitus after severe echovirus 9 infection: putative pathogenic pathways. *Diabetes* 49:1314–1318, 2000
36. Imagawa A, Hanafusa T, Miyagawa J, Matsuzawa Y: A novel subtype of type 1 diabetes mellitus characterized by a rapid onset and an absence of diabetes-related antibodies. *N Engl J Med* 3:343:301–307, 2000
37. Clements GB, Galbraith DN, Taylor KW: Coxsackie B virus infection and onset of childhood diabetes. *Lancet* 346:221–223, 1995
38. Szopa TM, Tichenter PA, Portwood ND, Taylor KW: Diabetes mellitus due to viruses –some recent developments. *Diabetologia* 36:687–695, 1993
39. Taylor LA, Carthy CM, Yang D, Saad K, Wong D, Schreiner G, Stanton LW, McManus BM: Host gene regulation during coxsackievirus B3 infection in mice; Assessment by microarrays. *Circ Res* 87:328–334, 2000