Coxsackievirus B1 Is Associated With Induction of β-Cell Autoimmunity That Portends Type 1 Diabetes

Running title: Coxsackievirus B1 and β-cell autoimmunity

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ABSTRACT

The rapidly increasing incidence of type 1 diabetes implies that environmental factors are involved in the pathogenesis. Enteroviruses are among the suspected environmental triggers of the disease, and the interest in exploring the possibilities to develop vaccines against these viruses has increased. Our objective was to identify enterovirus serotypes which could be involved in the initiation of the disease process by screening neutralizing antibodies against 41 different enterovirus types in a unique longitudinal sample series from a large prospective birth-cohort study. The study participants comprised 183 case children testing persistently positive for at least two diabetes-predictive autoantibodies and 366 autoantibody-negative matched control children. Coxsackievirus B1 was associated with an increased risk of β-cell autoimmunity. This risk was strongest when infection occurred a few months before autoantibodies appeared and it was attenuated by the presence of maternal antibodies against the virus. Two other Coxsackieviruses, B3 and B6, were associated with a reduced risk, with an interaction pattern suggesting immunological cross-protection against Coxsackievirus B1. These results support previous observations suggesting that the group B Coxsackieviruses are associated with the risk of type 1 diabetes. The clustering of the risk and protective viruses to this narrow phylogenetic lineage supports the biological plausibility of this phenomenon.

INTRODUCTION

Enteroviruses have been linked to type 1 diabetes in a number of previous studies (for reviews, see (1, 2)). The recent discovery of diabetes-associated polymorphisms in the innate immune system receptor for enteroviruses (IFIH1) has further increased the interest in the role of enterovirus infections in the pathogenesis of the disease. (3) However, this association has not been observed in all studies and the causal relationship has remained open.

More than 100 different enterovirus serotypes have been identified, which vary in their binding to various cellular receptors and in their ability to infect different cell types and organs. Consequently, different serotypes cause a diverse spectrum of diseases. The classical enterovirus disease, poliomyelitis, is caused by three serotypes, polioviruses 1, 2 and 3 which have a strong tropism for motoneurons in the spinal cord. This tropism is explained by the expression of the poliovirus receptor (PVR, CD166) on these cells. The virus spreads to the motoneurons in about 1% of infected individuals causing paralytic disease. Similarly, some other enteroviruses including the six Coxsackievirus B (CBV) serotypes seem to have a tropism for human pancreatic islets in vitro (4-7) and in vivo, (8-10) possibly because islet cells express the Coxsackie-adenovirus receptor (CAR), which is the major receptor for CBVs (11). The identification of the enterovirus serotypes which may induce the disease process leading to type 1 diabetes is important since it would enable further studies on the mechanisms of enterovirus-induced β-cell damage, and would pave the way for the development of a preventive vaccine. The lack of this information could also explain the variable results from previous studies which have been based on assays detecting several different enterovirus types as a group (2, 12). Despite the importance of this topic, large-scale systematic studies aimed at identifying diabetogenic enterovirus serotypes have not been performed. Previous reports, which have been based on case reports and small patient series, suggest that the CBV group

viruses may include diabetogenic serotypes (*1*), but also certain echovirus serotypes have been linked to type 1 diabetes. (13)

Here, the role of enterovirus infections was studied using the birth cohort samples systematically collected in the prospective Diabetes Prediction and Prevention (DIPP) study in Finland. By screening for the presence of neutralizing antibodies directed against a panel of 41 enterovirus serotypes, we assessed the association between each individual serotype and the appearance of diabetes-predictive autoantibodies. This made it possible to study the time-relationship between infection and initiation of the autoimmune process. This is the first large and systematic study aimed at the identification of diabetogenic enterovirus types at the time when the process appears to start.

RESEARCH DESIGN AND METHODS

Subjects

The study population was derived from the DIPP study. (14) Families with children carrying increased genetic risk for type 1 diabetes, defined by cord-blood HLA typing, were invited to participate in prospective follow-up starting from birth. Blood samples were drawn at the ages of 3, 6, 12, 18, and 24 months and once a year thereafter. All follow-up samples were screened for islet cell antibodies (ICA), and if a child seroconverted to positivity for ICA, autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA), and the tyrosine phosphatase-related insulinoma-associated 2 molecule (IA-2A) were also analyzed from all follow-up samples. Written consent was obtained from each family whose child took part, and the study was approved by the Ethical Committees of the Pirkanmaa Hospital and the Northern Ostrobothnia Hospital districts.

Our study was a nested case-control study (Figure 1) using the following criteria to select case and control children. First, case children had turned permanently positive for two or more diabetes-predictive autoantibodies and/or progressed to clinical type 1 diabetes. Two control children were selected for each case child. They all remained non-diabetic and autoantibody-negative for at least 2

years following the earliest detection of autoantibodies in the corresponding case child and were matched for time of birth (± 1 month except in 12 children ± 2 months), gender (60% were boys), HLA-DQB1 genotype and region. The final study cohort included 183 case and 366 control children born during the period from 1995 to 2006 and having an average age of 31 months (range 5-122 months) at initial seroconversion to autoantibody positivity (Supplementary Tables 1-2). One hundred nineteen case children progressed to type 1 diabetes by the end of July 2011.

HLA Genotyping

An analysis of the HLA-DQB1 genotype was performed from cord blood to identify selected alleles (DQB1*02, *03:01, *03:02, and *06:02/3) associated with susceptibility to or protection against type 1 diabetes. (15) The genotyping was based on hybridization with lanthanide-labeled oligonucleotide probes detected with time-resolved fluorometry. (16) Families with an infant carrying the high-risk HLA-DQB1*02/DQB1*0302 genotype or the moderate-risk DQB1*0302/x genotype (x ≠DQB1*03:02, *06:02, or*06:03) were invited for follow-up (Supplementary Table 3).

Detection of β-cell autoimmunity and clinical type 1 diabetes

ICA were detected by indirect immunofluorescence, while the three other autoantibodies were quantified with radiolabel binding assays. (17) We used cut-off limits for positivity of 2·5 Juvenile Diabetes Foundation units for ICA, 3·48 relative units (RU) for IAA, 5·36 RU for GADA (full-length GAD65, aa 1-585, used as construct), and 0·43 RU for IA-2A (the intracellular portion of the IA-2 molecule, aa 605-979, used as construct) representing the 99th percentile in more than 350 Finnish children. The disease sensitivity and specificity of the ICA assay were 100% and 98% in the fourth round of the International Workshops on Standardization of the ICA assay. The disease sensitivity of the IAA assay was 58% and the specificity 100% in the 2005 Diabetes Autoantibody Standardization Program Workshop. The same characteristics of the GADA assay were 82% and

96% and those of the IA-2A assay 72% and 100%, respectively. The diagnosis of type 1 diabetes was based on the WHO criteria.

Cells

Viruses were isolated, cultivated and seroneutralization assays were performed using the following cell lines: A549, Vero, RD, and GMK, of which the three first cell lines were purchased from ATCC whereas GMK was acquired from the National Institute for Health and Welfare, Finland.

Neutralizing antibodies against various enterovirus serotypes

Neutralizing antibodies were measured in serum or plasma against a total of 44 enterovirus strains representing 41 serotypes. Most of these viruses were isolated from DIPP children and from hospital patients in Finland and Sweden. All strains were plaque purified and sequenced in their VP1 region for serotyping. (18) Most of the viruses were analyzed using a standard plaque neutralization assay, (19, 20) while viruses that did not form clear plaques were analyzed using a microneutralization assay (Supplementary Table 4). All samples were screened using 1:4 and 1:16 dilutions. Inhibition was considered to be significant when the serum reduced the number of plaques more than 75% (plaque assay) or inhibited the ability of the virus to kill cells (microneutralization assay).

The identification of diabetogenic serotypes was based on a step-wise strategy (Figure 1). First, the neutralizing antibodies were analyzed in the samples where autoantibodies were detected for the first time in case children and in the corresponding samples in control children (cross-sectional analysis). All samples showing titers greater or equal to 1:4 were considered positive. In the next step, neutralizing antibodies were screened in samples from earlier time points (longitudinal analyses) for those enterovirus serotypes which were associated with diabetes risk at the cross-sectional primary screening step. These longitudinal analyses made it possible to diagnose infections by virus antibody seroconversions observed between two consecutive follow-up samples

(Supplementary Fig. 1). These earlier time points included samples taken 6 and 12 months before the initial seroconversion to autoantibody positivity (Fig. 1). The mean age and age range at these time points are shown in Supplementary Table 5. Some samples were collected from the children at such a young age, that they possibly contained maternal antibodies. Cord blood samples were therefore analyzed in these children, and when the presence of maternal antibodies could bias a positive result the sample was considered negative. In addition, cord blood samples and samples taken at the age of 18 months were analyzed from all children for CBV1 antibodies. The following definitions were used to diagnose an acute infection: the main definition was based on "Sensitive diagnostic criteria" where both transient and permanent antibody seroconversions were counted (if the child had serial transient seroconversions against the same virus only the first one was counted). The results were confirmed using more strict "Specific diagnostic criteria" where acute infections were diagnosed by the following criteria: a seroconversion from titer < 1:4 (seronegative) to 1:4 or higher (seropositive), a titer of 1:16 in at least one of the following samples and all subsequent samples positive.

Statistical Analyses

The primary analysis method was conditional logistic regression using the one-to-two age, gender, HLA and region matched case-control triplets. Data from matched case-control pairs and triplets were analyzed using Stata 8·2 (StataCorp, College Station, TX, USA) which allows for variable matching ratios of cases to controls. Conditional logistic regression was used to estimate the odds ratios (OR) with exact 95% confidence intervals (CI), and two-sided *P* values for univariate point estimates and multivariate modeling, to assess the association between enterovirus antibodies and diabetes-predictive autoantibodies. In the first phase, a cross-sectional analysis was carried out using data on the prevalence of enterovirus serotypes at the time point when the first diabetes-predictive autoantibodies were detected. The duration of exclusive and total breast-feeding was entered into a multivariate analysis to estimate adjusted odds ratios. Second, to study the temporal

profile of the associations detected in these cross-sectional analyses, infections occurring during all longitudinal time points before the detection of predictive autoantibodies were analyzed. The time was classified into three periods (simultaneously with the first detection of autoantibodies, 6 months before autoantibodies and 12 months or longer before autoantibodies), and the infections were diagnosed using both the sensitive and specific criteria described above. Third, the impact of the chronology of infections caused by different serotypes on the risk of β-cell autoimmunity was analyzed using the longitudinal data. Fourth, interactions between different serotypes were analyzed by studying the effect of different virus combinations. In addition to the raw *P* values the *P* values which have been corrected for the number of comparisons made (Bonferroni's correction) are presented.

RESULTS

Seroprevalences of coxsackievirus B1, B3 and B6 show a cross-sectional association with the risk to develop autoantibodies

Neutralizing antibodies were initially screened against 41 enterovirus serotypes in the first sample positive for diabetes-predictive autoantibodies. The conditional logistic regression analyses showed that CBV1 antibodies were more frequent in the cases than in the control children (59.0% vs. 50.1%; OR 1.5, 95% CI 1.0-2.2; *P*=0.04) suggesting that an infection with this enterovirus is associated with an increased risk of β-cell autoimmunity (Table 1). The statistical significance disappears if the *P* value is multiplied by the number of tested serotypes (N=41). The high seroprevalence of CBV1 in the control children (50.1%) indicates that this enterovirus is a common serotype in the population studied. Only one case and one control child were negative for all 41 tested enterovirus serotypes (median number of positive serotypes was nine in both groups).

Neutralizing antibodies to two closely related serotypes, CBV3 and CBV6, were less frequent in cases than in control children indicating a strong protective association for CBV3 (5.8% vs. 12.8%; OR 0.4, 95% CI 0.2-0.8; *P*=0·01) and a weaker protective association for CBV6 (26.6% vs. 35.3%;

OR 0.6, 95% CI 0.4-1.0; *P*=0.04) (Table 1). As above, the statistical significance disappears if these *P* values are multiplied by the number of tested serotypes. However, the fact that the protective serotypes were the closest genetically to CBV1 (Fig. 2), and no protective association was seen for more distant strains among the 41 analyzed, suggests that these findings reflect a true biological phenomenon. In fact, they support the plausible hypothesis that there is some immunological cross-protection between these closely related enterovirus types. The analysis of potential interactions between CBV1 and the other CBV serotypes indicated a clear risk effect when the child had experienced CBV1 alone without these protective serotypes (OR 2.5, 95% CI 1.4-4.7; *P*=0.003) while children infected by both CBV1 and one or more of the protective serotypes were not at risk (Table 2 and Supplementary Table 6).

The risk association of CVB1 and the protective association of CBV3 and CBV6 were also seen in the sub-cohort of 119 children who progressed to clinical type 1 diabetes (OR for CBV1 was 1.8, 95% CI 1.1-2.9; *P*=0.025), both among boys and girls, and in different age groups (data not shown). The effects of CBV1 and CBV3 remained significant after adjustment for the duration of breast-feeding and the number of older siblings while the effect of CBV6 became non-significant (a clear trend was observed also for CBV6, Supplementary Table 7).

The coxsackievirus B1 risk association was confirmed in longitudinal analyses before the appearance of the first autoantibodies in case children

The timing of infection with CBV1 was further assessed in a longitudinal analysis by detecting seroconversions in the neutralizing antibodies between consecutive follow-up samples collected before the first autoantibody-positive sample. The results showed an increased risk of autoantibody positivity when a CBV1 infection preceded the autoantibody appearance (Table 3). This association was strongest when CBV1 infections preceded the first autoantibody-positive sample by a few months, and it was observed using both the sensitive and strict infection criteria. The association was also seen in the subgroup of children who progressed to clinical type 1 diabetes.

Chronological order of coxsackievirus B infections

When the longitudinal data were analyzed to study the impact of the order of infections with CBV1 and the protective CBV serotypes, some trends suggesting a potential order effect were observed. When CBV1 was the first infecting serotype to occur, the children were at risk of developing autoantibodies whereas when CBV3 or CBV6 infection occurred first, the risk of developing autoantibodies was lower (Supplementary Table 8). This again supports the conclusion that infection by CBV3 or CBV6 provides some immunological protection from the diabetogenic effect of CBV1.

Maternal antibodies modulate the risk effect of coxsackievirus B1

The cord blood samples and samples taken at the age of 18 months were analyzed in order to explore whether protective maternal CBV1 antibodies in cord blood can modulate the risk association of CBV1 infections in young infants. The risk association was found to be strongest in the group who experienced CBV1 without maternal CBV1 antibodies (2.6; 95% CI 1.1-5.9; *P*=0.02) (Table 4).

DISCUSSION

This case-control study nested in the DIPP birth cohort is the first systematic study aimed at identifying enterovirus subtypes possibly associated with the induction of β-cell autoantibodies. The study has several unique strengths. First, it is based on the analysis of neutralizing antibodies which is the most reliable way to diagnose prior infection caused by a given enterovirus serotype. Second, it covers a large number of different serotypes (N=41), most of which represent wild-type

strains circulating in the background population. Third, it was performed in a prospective birth cohort study including a longitudinal sample series starting from cord blood which allowed the timing of the infections to be determined in relation to the time when autoantibodies first appeared. Fourth, the case and control subjects were matched for the most relevant potential confounders such as HLA-defined diabetes risk, gender, time of birth, age at sampling and the area of residence. Finally, the results provided by the cross-sectional and longitudinal analyses using different infection criteria were coherent.

We believe that the finding that the three serotypes identified are closely related phylogenetically (Fig. 2) is very significant. Indeed, if the signals detected in this study were due to arbitrary random noise in the methods, it would be unlikely that they would cluster together phylogenetically. Close clustering on the other hand, is precisely what would be expected for serotypes that could be either causative or protective, based on the highly plausible hypothesis of some degree of immunological cross-protection as discussed below.

The outcome reported here is consistent with the diabetogenic role of enteroviruses postulated in the literature and with predictions that can be made in searching for diabetogenic viruses. Prospective studies have shown that the autoimmune process usually begins at an early age (<3 years) (20, 21) and autoantibodies appear annually in “epidemic” peaks. (20) Consequently, the causative agent is probably frequent in the background population circulating continuously in very young children. The epidemiology of CBV1 fits with these predictions. CBV1 has been one of the most frequent enteroviruses isolated in recent years in the U.S. (22, 23) as well as in Korea (24), India, (25) Tunisia, (26) Western Germany, (27) and in Finland. (28) It can cause severe systemic infections in young infants, (29, 30) and it infects human pancreatic islets *in vitro* being one of the most cytolytic enterovirus serotype in this model. (7) In fact, insulitis and islet cell damage have been described in infants who have died of CBV1 infection. (31) Certain CBV1 strains induce also persistent infections in mice leading to chronic inflammatory myopathy. (32) Based on the generated data one can estimate that less than 5% of CBV1 infected children go on to develop type

1 diabetes. This fits with the low attack rate typical for enterovirus diseases. For example, in the beginning of the 20th century almost the whole population became infected by polioviruses but less than 1% of them developed motor neuron damage and paralysis. This implies also that the odds ratios obtained from serological screening studies remain relatively modest, even though CBV1 infection may explain the majority of the cases.

Surprisingly, the current study revealed that infections by two other CBVs, CBV3 and CBV6, were associated with a decreased risk of β-cell autoimmunity. A possible protective effect of CBV3 has actually been reported in a smaller study where patients with newly diagnosed type 1 diabetes were found to be less frequently positive for neutralizing antibodies against this serotype than control subjects. (*26*) This phenomenon could be explained by immunological cross-protection induced by CBV3 and CBV6 against the diabetogenic effect of CBV1. Such cross-protection, most likely due to cell-mediated immunity, has been reported in other virus diseases, e.g. between different rotavirus, papillomavirus and poliovirus types. (33-37) Cross-protection is also supported by the increased CBV1-related risk in children who were infected by CBV1 but none of the protective serotypes. Prevention of lethal CBV1 infection by a prior CBV3 infection has also been observed in a mouse model fitting nicely with the findings in the current study. (38) In addition to cross-protection, other mechanisms related to the induction of β-cell tolerance may mediate the protective effect of viruses against type 1 diabetes as described in NOD mice. (39, 40) In both cases, the close relationship between the protective and the diabetogenic serotypes suggests a particular impact of the CBV group enteroviruses on the risk of diabetes. Since CBVs are the only enteroviruses to use CAR, it can be hypothesized that they share some specific characteristics in terms of antigenicity and/or tropism.

Despite its virtues, the present study has also limitations. The first relates to the population studied being exclusively from Finland and covering a relatively limited 10-year period. Consequently, we cannot exclude a timing effect of CBV1 infections or a strain-specific effect of this serotype. A timing effect could also explain the low prevalence of CBV4 serotype which has

been linked to type 1 diabetes in previous studies. Accordingly, it will be important to confirm these findings in other populations. The statistical power of the current study allowed the identification of viruses with major risk effects while viruses with weaker effects may have been missed. Adding new data sets would also help to assess further the combined effect of the three identified CBV serotypes. The virus strains used in the neutralization assay represent the most common enterovirus serotypes, (22, 23) but they do not include all serotypes known today (many of them are also difficult to cultivate and to produce cytopatic effect in vitro). Therefore we cannot exclude the possibility that other risk or protective serotypes may have been missed.

The current findings have aspects which fit with causality. First, the CBV1–related risk effect showed logical time relationship - it preceded the initiation of the autoimmune process. In addition, CBV1 infections peaked a few months before autoantibodies first appeared, which overlaps with the previously observed peak in the frequency of enterovirus RNA in serum, (41) fitting with the rapid induction of islet autoantibodies in enterovirus-infected mice. (42) Second, the accumulation of both risk and protective viruses to a small subgroup of phylogenically close enteroviruses supports the biological relevance of our findings. Third, the discovery of protective viruses fits with immunological cross-protection attenuating infections caused by closely related viruses. Fourth, the observation that maternal CBV1 antibodies modulated the risk effect of CBV1 supports biological plausibility since maternal antibodies protect the child against enterovirus infections (43) (44). Finally, we have observed a similar risk effect of CBV1 in another study where neutralizing antibodies were analyzed in patients with newly diagnosed type 1 diabetes and control subjects in five European countries (41).

In summary, the results are in line with the previous literature suggesting a link between enterovirus infections and type 1 diabetes. The identification of CBV1 as a potentially diabetogenic virus type is a new discovery which offers possibilities to explore the mechanisms of enterovirus-induced diabetes and may also open the door for the development of an enterovirus vaccine against the disease. Further studies are needed to confirm these findings in other populations. The

identification of serotypes with opposite effects on type 1 diabetes implies that serotype-specific methods should be used in such studies.

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Author contribution

HeHy had full access to all the data in the study and takes responsibility of the integrity of the data and the accuracy of the data analysis. The study was designed by the steering group including HeHy, MK, JA and VL. MK, RV, OS, JI and HeHy participated in the recruitment of children to the DIPP study and OHL, HaHo, SO, MK, OP, MMP, TR, RH, and HeHy in the virus analyses. SMV

was responsible for the dietary data (breastfeeding). Data analyses were carried out by HeHu, PA and JL. All authors contributed to the data interpretation and the preparation of the manuscript. HaHo is the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Competing interests

HeHy and MK are minor (<5%) shareholders and members of the board of Vactech Ltd., which develops vaccines against picornaviruses. Other authors declare no potential conflicts of interest.

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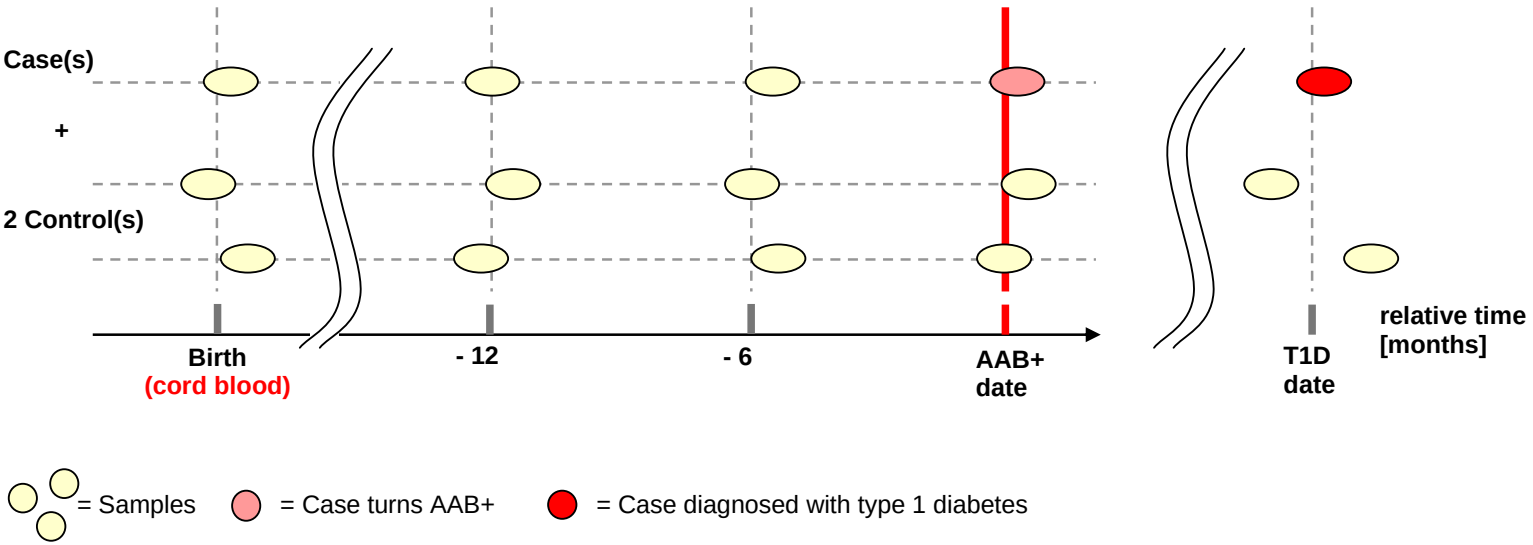


FIG. 1. Study setup.

The nested study consisted of 183 case/control triplets in which for each case child fulfilling the defined criteria two matched controls were selected. First, the neutralizing antibodies were analyzed in the samples where autoantibodies were detected for the first time (AAB+ date sample) in case children and in the corresponding samples in control children (cross-sectional analysis). Next, neutralizing antibodies were screened in samples from earlier time points (6 months and 12 months before AAB+ date) and in samples taken at birth (cord blood) at the age of 18 months to carry out longitudinal analyses for those enterovirus serotypes which were associated with the modulated diabetes risk at the cross-sectional primary screening step. However, the complete set of follow-up samples was not available from every child, which explains the small variation in the number of samples in different analyses. The information on the diagnosis of type 1 diabetes (T1D date) was utilized to run sub-cohort analyses for those triplets in which the case progressed to type 1 diabetes.

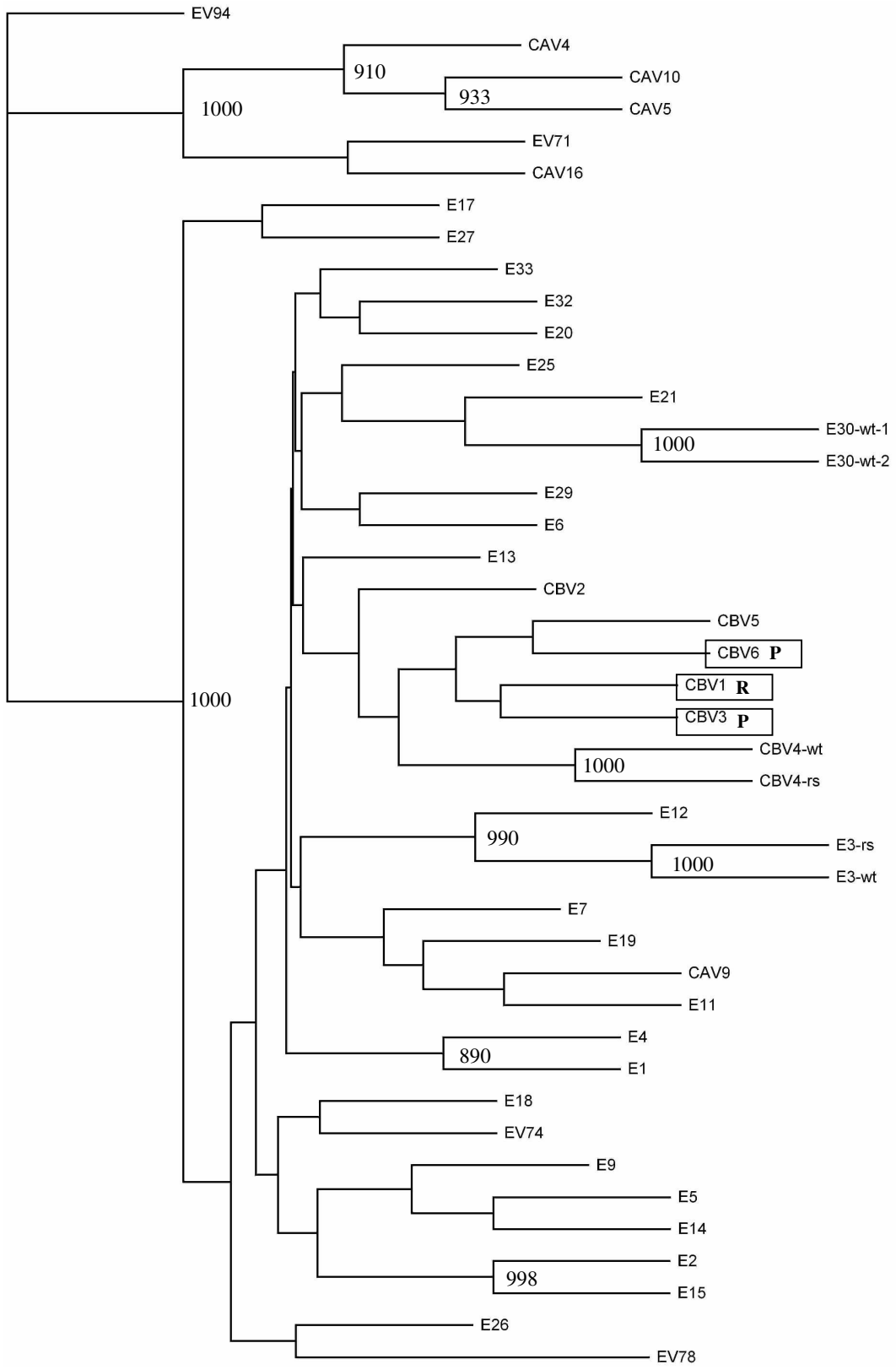


Figure 2. Consensus phylogenetic tree of the 44 virus strains based on 104 amino acids of the VP1 region.

The part of VP1 region of all 44 viruses was sequenced and the obtained sequences were blasted against the NCBI non-redundant nucleotide database. Phylogenetic analysis was done using Phylip program package version 3.69 (Felsenstein, J. 1993. PHYLIP: phylogeny inference package, version 3.69. University of Washington, Seattle). The phylogenetic tree was constructed using the Protdist program with the parameters of the Kimura 2 model and the amino acid matrix was processed with the Kitsch program. The consensus tree was treated with the Consense program. This analysis implies a close genetic relationship of the three CBV viruses which were associated with β-cell autoimmunity.

Abbreviations of virus strains: E = Echovirus, CAV = Coxsackie virus A, CBV = Coxsackie virus B and EV = Enterovirus. Risk-associated CBV1 strain is indicated by R and protective CBV3 and CBV6 strains by P. The bootstrap confidence levels were analyzed with 1000 pseudoreplicate data sets and higher than 70% bootstrap levels were plotted onto the tree.

TABLE 1.

Odds ratios for the association between neutralizing antibodies to 44 enteroviruses (41 serotypes) and signs of progressive β-cell autoimmunity (positivity for ≥2 diabetes-predictive autoantibodies) in case (N=183) and matched control (N=366) children.

	Nab prevalence				
VIRUS	**% case**	**% control**	**OR**	**95% CI**	***P* value**
CAV4	28.7	31.7	0.9	(0.6-1.3)	.46
CAV5	15.0	15.0	1.0	(0.6-1.9)	.96
CAV6	17.1	14.7	1.2	(0.7-2.1)	.44
CAV10	69.8	61.8	1.4	(0.8-2.5)	.27
CAV16	12.5	16.6	0.7	(0.4-1.2)	.23
EV71	8.4	7.9	1.1	(0.5-2.4)	.89
CAV9	7.5	8.4	0.9	(0.4-1.8)	.72
CBV1	**59.0**	**50.1**	**1.5**	**(1.0-2.2)**	**.04**
CBV2	46.6	48.8	0.9	(0.6-1.3)	.61
CBV3	**5.8**	**12.8**	**0.4**	**(0.2-0.8)**	**.01**
CBV4-wt*	5.2	8.1	0.6	(0.2-1.3)	.19
CBV4-rs#	5.2	7.2	0.7	(0.3-1.5)	.34
CBV5	7.5	7.8	0.9	(0.4-1.9)	.81
CBV6	**26.6**	**35.3**	**0.6**	**(0.4-1.0)**	**.04**
E1	25.9	26.2	0.6	(0.3-1.3)	.19
E2	9.1	9.9	1.0	(0.5-1.9)	.95
E3-wt	5.2	4.1	1.3	(0.6-3.2)	.54
E3-rs	43.8	39.9	1.2	(0.8-1.8)	.33
E4	1.2	1.5	0.8	(0.2-4.1)	.79
E5	36.0	37.2	0.9	(0.6-1.5)	.73
E6	8.6	7.5	1.2	(0.6-2.3)	.65
E7	18.4	17.9	1.0	(0.6-1.7)	.90
E9	7.6	9.8	0.7	(0.3-1.5)	.34
E11	32.4	36.3	0.8	(0.5-1.2)	.33
E12	36.8	31.7	1.3	(0.9-2.0)	.22
E13	2.3	4.1	0.5	(0.2-1.7)	.30
E14	7.6	5.6	1.3	(0.6-2.9)	.43
E15	8.7	13.0	0.6	(0.3-1.2)	.13
E17	5.8	7.8	0.7	(0.3-1.5)	.31
E18	3.5	3.8	0.9	(0.3-2.5)	.87
E19	9.6	13.1	0.7	(0.3-1.5)	.34
E20	6.4	5.2	1.3	(0.6-2.8)	.50
E21	28	32.4	0.8	(0.5-1.2)	.29
E25	6.4	4.3	1.5	(0.7-3.2)	.34
E26	1.7	3.5	0.5	(0.1-1.8)	.27
E27	5.8	6.5	0.9	(0.4-1.9)	.75
E29	9.3	7.4	1.3	(0.6-2.8)	.45

E30-wt-1	98.0	96.8	0.8	(0.2-3.8)	.79
E30-wt-2	72.3	76.1	0.8	(0.5-1.3)	.38
E32	43.9	43.2	1.0	(0.6-1.6)	.97
E33	81.9	80.7	1.2	(0.7-1.8)	.56
EV74	60.1	59.6	1.0	(0.7-1.5)	.97
EV78	2.3	4.4	0.5	(0.2-1.6)	.25
EV94	5.3	5.0	1.1	(0.7-3.1)	.93

*wt = wild type strain
[#]RS = reference strain

% case represents the antibody prevalence in case children and % control the prevalence in control children.
OR = odds ratio: 95% CI = 95% confidence interval

TABLE 2

Association of different combinations of risk- and protective-type coxsackievirus B infections with the risk of β-cell autoimmunity as defined by virus antibody positivity at the time of autoantibody seroconversion (cross-sectional analysis among 180 case and 360 matched control children).

Antibodies against risk serotype	**Antibodies against protective serotypes**	**OR**	**95% CI**	***P* value**
CBV1 neg	CBV3 or CBV6 pos	1	ref	
CBV1 neg	CBV3 and CBV6 neg	1.6	(0.9-3.1)	.12
CBV1 pos	CBV3 or CBV6 pos	1.5	(0.8-2.9)	.20
CBV1 pos	CBV3 and CBV6 neg	**2.5**	**(1.4-4.7)**	**.003**

The reference group comprises children with the lowest predicted risk being seropositive for the protective serotypes but not for CBV1. OR = odds ratio; 95% CI = 95% confidence interval

TABLE 3

The risk for β-cell autoimmunity associated with Coxsackievirus B1 infections according to the time when they were diagnosed in 183 case and 366 matched control children (119 case children who progressed to clinical type 1 diabetes and their 239 controls were included in the diabetes subgroup).

TIMING OF CBV1 INFECTION*	**Sensitive diagnostic criteria**			**Specific diagnostic criteria**		
Whole nested case-control series	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**
No infection	1	ref		1	ref	
12 months or longer before autoantibodies	1.3	(0.8-2.3)	0.33	1.0	(0.5-2.2)	0.93
6 months before autoantibodies	**2.0**	**(1.1-3.6)**	**0.03**	1.9	(0.7-5.2)	0.23
Simultaneously with autoantibodies	1.5	(0.9-2.4)	0.11	**2.1**	**(1.0-4.4)**	**0.04**
Case children who progressed to type 1 diabetes and their controls						
No infection	1	ref		1	ref	
12 months or longer before autoantibodies	1.0	(0.4-2.2)	0.91	0.7	(0.2-2.0)	0.48
6 months before autoantibodies	**2.0**	**(1.0-4.2)**	**0.05**	1.8	(0.6-5.0)	0.27
Simultaneously with autoantibodies	1.6	(0.89-2.9)	0.11	**2.5**	**(1.1-5.6)**	**0.03**

*Average time in relation to autoantibody seroconversion

The sensitive and specific diagnostic criteria analyses were performed as defined in the Methods section. OR = odds ratio; 95% CI = 95% confidence interval.

TABLE 4

The risk of β-cell autoimmunity in children according to their exposure to CBV1 by the age of 18 months (CBV1 seropositive at that age) and presence of protective CBV1 antibodies in cord-blood among 127 case and 254 matched control children.

CBV1 seropositivity		**Observed risk of β-cell autoimmunity**		**Expected risk of β-cell autoimmunity**[***]
Cord-blood	**18 months**	**OR and 95% CI**	***P* value**	
pos*	neg**	1 (reference)		Lowest
neg	neg**	1.6 (0.7-3.9)	0.28	Low
pos*	pos	2.1 (0.8-5.6)	0.12	High
neg	pos	**2.6 (1.1-5.9)**	**0.02**	Highest

* only antibody titers 16 or higher were considered positive in cord-blood since low antibody levels disappear rapidly from the child's circulation.

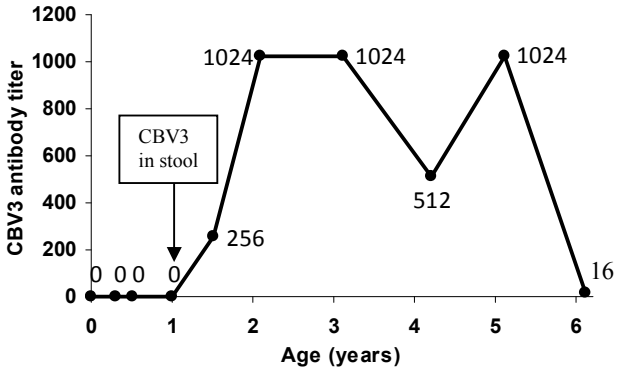
**negative antibody result does not exclude early CBV1 infection due to possible transient antibody responses in these very young infants

[***]Expected risk refers to theoretical risk predicted on the basis of CBV1 seropositivity in cord blood (maternal antibodies) and at the age of 18 months.

OR = odds ratio; 95% CI = 95% confidence interval

SUPPLEMENTARY MATERIALS

Supplementary figures



Supplementary Figure 1. Neutralizing antibody response induced by coxsackievirus B3 (CBV3) infection in one DIPP child. CBV3 was detected in a stool sample taken at the age of 1.2 years. Neutralizing antibody titers against CBV3 are shown for all follow-up serum samples.

Supplementary tables

Supplementary Table 1. Distribution according to year of birth in the children taking part in the study

Year of birth	No of participants
1995	9
1996	25
1997	39
1998	86
1999	90
2000	71
2001	58
2002	45
2003	73
2004	31
2005	19
2006	3

Supplementary Table 2. Distribution of the sampling age (in months) at the time when the first autoantibody positive sample was drawn in the case children

Age in months	4	6	7	9	11	12	13	14	17	18	19	20	21	23	24	25	26	35	36	37
Number of children	1	2	2	1	2	31	8	3	3	20	8	1	1	1	15	4	2	7	14	9

Age in months	38	39	42	46	47	48	49	50	52	60	61	62	72	73	74	84	95	96	98	121
Number of children	1	2	1	2	3	7	5	4	1	6	2	3	2	2	1	1	1	2	1	1

Supplementary Table 3. Distribution of HLA genotypes among the study subjects. Children carrying the HLA genotype DQB1*02/DQB1*0302 were categorized into the high-risk group and children with the DQB1*0302/x Genotype (x≠DQB1*0301 or *0602) into the moderate risk group

HLA DQB1 genotype	Number of children	Percentage
*02/*0302	154	28.1
*0302/x	395	71.9
Total	**549**	**100**

Supplementary Table 4. Summary of enterovirus strains, their source, seroneutralization method, and cell lines used in the virus isolation and cultivation and the measurement of neutralizing antibodies against different enterovirus serotypes

No	Virus isolate	Source of the virus	Strain†	Neutralization method	Cell line used in neutralization assay
1	CAV4	DIPP	isolate 10433	plaque	RD
2	CAV5	DIPP	isolate P-550/CA5/Kanagawa/2000	MN	RD
3	CAV6	DIPP	isolate CSF-1739/07 VP1	MN	RD
4	CAV10	DIPP	P-2206/CA10/Kanagawa/2003	MN	RD
5	CAV16	DIPP	W42-44/01	MN	RD
6	EV71	DIPP	isolate 03784-MAA-97	MN	Vero
7	CAV9	DIPP	FR-08-2005-149	plaque	GMK
8	CBV1	HUSLAB	isolate CVB1Nm	plaque	GMK
9	CBV2	Laboratory center	FR-CASE4	plaque	GMK
10	CBV3	DIPP	CBV3-18219-02 from Moldova polyprotein	plaque	GMK
11	CBV4-wt#	DIPP	isolate P234pak92	plaque	GMK
12	CBV4-rs*	HUSLAB	Tuscany	plaque	GMK
13	CBV5	DIPP	isolate CVB5-CSF1841/BLR/2003	plaque	GMK
14	CBV6	ATCC	Schmitt [1-15-21]	plaque	GMK
15	Echo-1	Laboratory center	isolate 10429	plaque	GMK
16	Echo-2	Virology, University of Turku	152-77	plaque	GMK
17	Echo-3-wt	Virology, University of Tampere	PicoBank/DM1/E3	plaque	GMK
18	Echo-3-rs	HUSLAB	Morrisey	plaque	GMK
19	Echo-4	ATCC	Pesascek	plaque	GMK
20	Echo-5	ATCC	isolate Noyce	plaque	GMK
21	Echo-6	Virology, University of Tampere	Germany/120/2003	plaque	GMK
22	Echo-7	Laboratory center	FR-07-2000-55	plaque	GMK

23	Echo-9	Laboratory center	clone: No.66	plaque	GMK
24	Echo-11	DIPP	NET/2000-10025	plaque	GMK
25	Echo-12	Laboratory center	isolate: 120-98	plaque	GMK
26	Echo-13	DIPP	isolate FR-06-2000-93	plaque	GMK
27	Echo-14	ATCC	Tow	plaque	GMK
28	Echo-15	HUSLAB	CH6-51	plaque	GMK
29	Echo-17	ATCC	CHHE-29	plaque	GMK
30	Echo-18	HUSLAB	Metcalf	plaque	GMK
31	Echo-19	HUSLAB	isolate 87SD140	plaque	GMK
32	Echo-20	HUSLAB	isolate 10465	plaque	GMK
33	Echo-21	HUSLAB	Farina	plaque	GMK
34	Echo-25	Laboratory center	isolate SE-97-80688	plaque	GMK
35	Echo-26	HUSLAB	Coronel (11-3-6)	plaque	GMK
36	Echo-27	ATCC	Bacon	plaque	GMK
37	Echo-29	ATCC	JV-10	plaque	GMK
38	Echo-30-wt1	Laboratory center	isolate CF2191-01	plaque	GMK
39	Echo-30-wt2	Laboratory center	Bern7/ch1996	plaque	GMK
40	Echo-32	HUSLAB	PR 10	plaque	GMK
41	Echo-33	ATCC	Toluca-3	plaque	GMK
42	EV74	SMI	FRA99-130	plaque	GMK
43	EV78	SMI	Human enterovirus 78 polyprotein gene, partial cds Length=2574	MN	RD
44	EV94	SMI	isolate 19/04 from Democratic Republic of the Congo	MN	A594

#wt= field isolate
*rs = reference strain
†closest strain according to partial VP1-VP3 sequence used as blast search string
MN=microneutralization (Although microneutralization was used to assay these viruses actually all viruses formed plaques or plaque like structures seen under the microscope and therefore they could be isolated as single “plaques” for further cultivation.)
Laboratory center = Laboratory Center, Tampere University Hospital, Tampere, Finland
HUSLAB = Laboratory Center, Helsinki University Central Hospital, Helsinki, Finland
SMI = Smittskyddinstitutet, Stockholm, Sweden

Supplementary Table 5. The median age and quartiles (Q1 and Q3) in months of the children at the various sampling time points used for the analyses of neutralizing antibodies to different enterovirus strains

Sampling time point	Median	Q1	Q3
-12 months	24	9	36
-6 months	12	6	13
At seroconversion to autoantibody positivity	24	13	38

Supplementary Table 6. Effect of different combinations of risk and protective type Coxsackievirus B infections on the risk of β-cell autoimmunity at the time of autoantibody seroconversion (cross-sectional analysis). The reference group in this analysis comprises children with the lowest predicted risk being seropositive for one or more of the protective serotypes but seronegative for CBV1. Altogether 180 case children and 360 control children were used in these analyses. OR = Odds Ratio; 95% CI = 95% Confidence Interval

Risk serotype	Protective serotype	OR	95% CI	*P* value
CBV1	**CBV3 or CBV4 or CBV6***			
neg	pos	1	ref	
neg	neg	1.5	(0.8-2.7)	0.16
pos	pos	1.3	(0.7-2.3)	0.42
pos	neg	**2.6**	**(1.4-4.6)**	**0.001**
CBV1	**CBV3 or CBV6**			
neg	pos	1	ref	
neg	neg	1.6	(0.9-3.1)	0.12
pos	pos	1.5	(0.8-2.9)	0.20
pos	neg	**2.5**	**(1.4-4.7)**	**0.003**
CBV1	**CBV3**			
neg	pos	1	ref	
neg	neg	2.3	(0.8-7.0)	0.14
pos	pos	1.5	(0.4-5.8)	0.53
pos	neg	**3.6**	**(1.2-10.7)**	**0.02**
CBV1	**CBV6**			
neg	pos	1	ref	
neg	neg	1.4	(0.7-2.7)	0.28
pos	pos	1.6	(0.8-3.2)	0.22
pos	neg	**2.2**	**(1.1-4.1)**	**0.02**
CBV1	**CBV3 and CBV6**			
neg	pos	1	ref	
neg	neg	3.2	(0.4-26.7)	0.28
pos	pos	2.0	(0.18-23.3)	0.56
pos	neg	4.9	(0.6-40.4)	0.14

*This largest group of protective serotypes includes CBV4 since it showed a protective non-significant trend in primary antibody screening and belonged to the same phylogenetic group as CBV1, CBV3 and CBV6 which showed statistically significant effects.

Supplementary Table 7. Effect of CBV1, CBV3 and CBV6 infections on the risk of β-cell autoimmunity when adjusted for the duration of breast feeding (160 case and 317 control children) or the number of older siblings (177 case and 344 control children). Cross-sectional virus antibody analyses were performed at the time of autoantibody seroconversion. Odds ratios (OR) and 95% confidence intervals (95% CI) are adjusted for the duration of exclusive breast-feeding (time when the child has not received any other nutrients than breast-milk), overall duration of breast feeding (the total time when the child has received breast-milk) and number of older siblings at birth.

	Adjustment								
	Duration of exclusive breast-feeding			**Overall duration of breast-feeding**			**Number of older siblings**		
	OR	**95% Cl**	***P* value**	**OR**	**95% Cl**	***P* value**	**OR**	**95% Cl**	***P* value**
CBV1	**1.6**	**(1.1-2.5)**	**0.020**	**1.6**	**(1.0-2.4)**	**0.030**	**1.5**	**(1.0-2.3)**	**0.032**
CBV3	**0.3**	**(0.2-0.8)**	**0.008**	**0.3**	**(0.1-0.6)**	**0.002**	**0.3**	**(0.2-0.7)**	**0.005**
CBV6	0.7	(0.4-1.1)	0.088	0.7	(0.4-1.1)	0.092	0.8	(0.5-1.2)	0.288

Supplementary Table 8. The effect of the order of infection on the risk of β-cell autoimmunity. The reference group comprises children who had none of the CBV infections studied or who had been infected by the protective serotypes before being infected by CBV1. Infections were diagnosed by virus antibody seroconversion observed between consecutive follow-up samples taken before or at the sampling date of the first autoantibody positive sample. Altogether 183 case children and 366 control children were entered into these analyses. OR = Odds Ratio; 95% CI = 95% Confidence Interval

Protective serotype: CBV3, CBV4 or CBV6 * **Risk serotype: CBV1**	**OR**	**95% CI**	**P value**
Protective serotype first or negative for both protective serotype and CBV1	1	ref	
CBV1 first	**1.5**	**(1.0-2.2)**	**0.06**
CBV1 and protective serotype simultaneously**	1.2	(0.7-1.0)	0.54
Protective serotype: CBV3 or CBV6 **Risk serotype: CBV1**	**OR**	**95% CI**	**P value**
Protective serotype first or negative for both protective serotype and CBV1	1	ref	
CBV1 first	1.4	(0.9-2.1)	0.10
CBV1 and protective serotype simultaneously**	1.1	(0.7-1.8)	0.64
Protective serotype: CBV3 **Risk serotype: CBV1**	**OR**	**95% CI**	**P value**
Protective serotype first or negative for both protective serotype and CBV1	1	ref	
CBV1 first	**1.5**	**(1.0-2.2)**	**0.03**
CBV1 and protective serotype simultaneously**	1.4	(0.5-4.4)	0.53

*This largest group of protective serotypes includes CBV4 since it showed a protective non-significant trend in primary antibody screening and belonged to the same phylogenetic group as CBV1, CBV3 and CBV6 which showed statistically significant effects.
**In this category infections occurred in the same sample interval and therefore it is not possible to define their mutual time order.