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Nilsson, Anna-Lena; Vaziri Sani, Fariba; Broberg, Per; Elfaitouri, A; Pipkorn, R; Blomberg, J; Ivarsson, Sten; Larsson, Helena; Lernmark, Åke

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PO Box 117
221 00 Lund
+46 46-222 00 00

For J. Med. Virol.

**Serological Evaluation of Possible Exposure to Ljungan Virus and Related Parechovirus
in Autoimmune (Type 1) Diabetes Children and Controls**

**A-L. Nilsson^{1,2}, F. Vaziri-Sani², P. Broberg³, A. Elfaitouri⁴, R. Pipkorn⁵, J. Blomberg⁴,
S-A. Ivarsson², H. Elding Larsson² and Å. Lernmark²**

¹Department of Paediatrics, Östersund Hospital, Östersund, Sweden

²Department of Clinical Sciences, Skåne University Hospital, Lund University/CRC, Malmö, Sweden

³Department of Oncology and Cancer Epidemiology, Clinical Sciences, Lund University, Sweden

⁴Section of Clinical Microbiology, Department of Medical Sciences, Uppsala University, Sweden

⁵Deutsches Krebsforschungszentrum, Heidelberg, Germany

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Corresponding author:

Anna-Lena Nilsson MD

Department of Paediatrics

Östersund Hospital

SE-831 83 Östersund

Sweden

Phone: +46 63 153977. Fax: +46 63 154505

Abstract

Exposure to Ljungan virus (LV) is implicated in the risk of autoimmune (type 1) diabetes but possible contribution by other parechoviruses is not ruled out. The aim was to compare children diagnosed with type 1 diabetes in 2005-2011 (n=69) with healthy controls (n=294), all from the Jämtland County in Sweden, using an exploratory suspension multiplex immunoassay for IgM and IgG against 26 peptides of LV, human parechoviruses (HPeV), Aichi virus and poliovirus in relation to a radiobinding assay (RBA) for antibodies against LV and InfluenzaA/H1N1pdm09. Islet autoantibodies and HLA-DQ genotypes were also determined.

1) All five LV-peptide antibodies correlated to each other ($p<0.001$) in the suspension multiplex IgM- and IgG-antibody assay; 2) The LV-VP1₃₁₋₆₀-IgG correlated with insulin autoantibodies alone ($p=0.007$) and in combination with HLA-DQ8 overall ($p=0.022$) as well as with HLA-DQ 8/8 and 8/X subjects ($p=0.013$); 3) RBA detected LV antibodies correlated with young age at diagnosis ($p<0.001$) and with insulin autoantibodies ($p<0.001$) especially in young HLA-DQ8 subjects ($p=0.004$); 4) LV-peptide-VP1₃₁₋₆₀-IgG correlated to RBA LV antibodies ($p=0.009$); 5) HPeV3-peptide-IgM and -IgG showed inter-peptide correlations ($p<0.001$) but only HPeV3-VP1₁₋₃₀-IgG ($p<0.001$) and VP1₉₅₋₁₂₄-IgG ($p=0.009$) were related to RBA LV antibodies without relation to insulin autoantibody positivity ($p=0.072$ and $p=0.486$ respectively).

Both exploratory suspension multiplex IgG to LV-peptide VP1₃₁₋₆₀ and RBA detected LV antibodies correlated with insulin autoantibodies and HLA-DQ8 suggesting possible role in type 1 diabetes. It remains to be determined if cross-reactivity or concomitant exposure to LV and HPeV3 contributes to the seroprevalence.

Keywords:

Picornavirus

Autoimmune disease

HLA-DQ

Islet autoantibodies

Insulin autoantibodies

Background

Environmental factors may explain observations of temporal variations in type 1 diabetes incidence [Gamble, 1975; Hermann et al., 2003; Stefan et al., 2014]. HLA-DQ2 and -DQ8 risk haplotypes [Kockum et al., 1999; EURODIAB, 2000; Kukko et al., 2004] and also non-HLA genetic factors are likely to contribute to the high incidence in countries like Sweden [Graham et al., 2002; Smyth et al., 2006; Concannon et al., 2009]. The possible influence of viral infections as a trigger of islet autoimmunity, clinical onset of type 1 diabetes, or both has been reviewed [Graves et al., 1997; Kolehmainen et al., 2013]. Several studies have suggested that enterovirus may be associated with the appearance of islet autoimmunity, type 1 diabetes, or both [Lonnrot et al., 2000; Serreze et al., 2000; Cabrera-Rode et al., 2003].

Ljungan virus (LV), a *Parechovirus* possibly pathogenic to humans [Johansson et al., 2002; Ekstrom et al., 2007] has been suggested to contribute to type 1 diabetes [Lindberg and Johansson, 2002; Niklasson et al., 2003a]. LV was defined as a separate species in the *Parechovirus* genus [Lindberg and Johansson, 2002; Niklasson et al., 2003b]. Formerly classified as an *Enterovirus* the *Parechovirus* was later reclassified as a separate genus in the *Picornaviridae* family containing *Human Parechoviruses* (HPeV) and *Ljungan virus*. Type 1 and 2 HPeV, formerly known as *Echovirus* 22 and 23, respectively, have been associated with type 1 diabetes [Niklasson et al., 1998; Johansson et al., 2002].

LV was first isolated in a bank vole, *Myodes glareolus*, from the valley of the river Ljungan in northern Sweden [Niklasson et al., 2003b]. Bank voles captured in the wild developed diabetes and had not only antibodies against LV but also autoantibodies against glutamic acid decarboxylase, islet antigen-2 and insulin [Niklasson et al., 2003a]. The population of bank voles in northern Sweden fluctuates with multiannual population cycles showing similarities with the fluctuations of the incidence of type 1 diabetes [Niklasson et al., 2003b; Hornfeldt et

al., 2005]. Levels of LV antibodies were increased in young age-at-onset of type 1 diabetes children but a possible relationship to the incidence of type 1 diabetes could either not be determined [Niklasson et al., 2006] or was suggestive [Niklasson et al., 2003a]. In previous investigations high level LV antibodies were found to fluctuate with season and to correlate with type 1 diabetes indicating that past exposure to LV may be associated with type 1 diabetes [Nilsson et al., 2009; Nilsson et al., 2013].

The specificity of the LV antibody analysis was questioned as children are often infected by any of 16 known strains of HPeV and it could not be excluded that LV antibodies would be explained by reactivity with other viruses.

The aim of this study was to compare IgM- and IgG-antibodies to both LV and HPeV peptides detected in an exploratory indirect antibody capture immunoassay [Blomberg et al., 2012] with LV antibodies detected in a RBA [Niklasson et al., 2003a; Nilsson et al., 2009; Nilsson et al., 2013] and to test whether these LV antibodies were related to islet autoantibodies or to type 1 diabetes-associated HLA-DQ genotypes.

Materials and Methods

Serum samples were obtained from 72 (F/M 37/35) children, 1-18 (median 10.0; range 1.0-17.9) years of age, when diagnosed with type 1 diabetes in June 2005 - November 2010 in Jämtland County in Sweden. Healthy controls were 295 (F/M 158/137) children, 14 (median 14.1; range 12.7-15) year old school children also from Jämtland County. The control samples were collected in April 2009 – January 2011 at separate occasions; April, October and November 2009, May, November and December 2010 and January 2011.

The exploratory suspension multiplex immunoassay analyses were performed on samples from 69 of the type 1 diabetes children and 294 of the controls.

All samples were obtained after informed consent from patients and parents and the study was approved by the Research Ethics Committee, University of Umeå, Umeå, Sweden.

There are no financial or other conflicts of interest for any of the authors.

Ljungan virus antibody RBA

Coupled *in vitro* transcription translation of the *Ljungan virus* isolate 87-012 representing 812 amino acids (6-817) of the LV-1 protein [Johansson et al., 2002] in a pCRII-TOPO vector [Nilsson et al., 2009; Nilsson et al., 2013] was performed in a reaction mixture containing 2 µg of pCRII-TOPO vector, 50 µl TNT® rabbit reticulocyte lysate, 4 µl TNT® reaction buffer, 2 µl amino acid mixture without methionine, 2 µl RNasin® Ribonuclease inhibitor, 2 µl SP6 RNA Polymerase, (all from Promega Corporation, Madison, WI, USA), 4 µl 35S-methionine (N1000 Ci/mmol, Amersham, Buckinghamshire, UK) and nuclease-free water to a final volume of 100 µl. The reaction mixture was incubated for 90 min at 30 °C with shaking (300 rpm in an Eppendorf Thermomixer, Eppendorf, Hamburg, Germany). The translation product

was immediately subjected to gel filtration on Illustra™ NAP-5 Columns (GE Healthcare Bio-Sciences, Uppsala, Sweden) and radioactivity incorporated into protein was determined (1450 MicroBeta TriLux Microplate Scintillation-Luminescence Counter, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA).

The LV antibody RBA was carried out in V-formed 96-well plates (catalog nr 442587, Micro Well™ plates, Nunc A/S, Roskilde, Denmark) over night at 4°C in duplicate samples of 2.5 µl of serum incubated with 60 µl labeled antigen at final concentration of 425±25 cpm/µl after dilution in antigen buffer (150 mmol/l NaCl, 20 mmol/ Tris, pH 7.4, 0.15%, v/v, Tween 20, 0.1%, w/v, BSA). A total of 50 µl reaction mixture was then incubated for 1 h at 4 °C with 50 µl 20% Protein A Sepharose 4B (PAS, Catalog nr 101042, Invitrogen Corporation, Camarillo, CA, USA), washed five times at 4°C in a 96-well MultiScreen-DV filtration plate (catalog nr MSDVN6B50, Merck Millipore Corporation, Billerica, MA, USA). The plates were then washed 8 times in wash buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 0.15% Tween 20) using a Multiscreen vacuum manifold (Multi-Screen^{HTS}, Vacuum Manifold, Millipore, Merck KGaA, Darmstadt, Germany). Antibody-bound radioactivity was counted in a β-counter (1450 MicroBeta TriLux Microplate Scintillation-Luminescence Counter Catalog nr 1450-021, PerkinElmer, San Jose, CA, USA). Sepharose-bound radioactivity was converted in all runs into in-house units (U) using individual standard curves generated by six step doubling dilutions of high-titer guinea pig antisera (Antiserum 174 F #4) [Niklasson et al., 2003a] [Niklasson et al., 2003b]. Intra-assay CV for duplicate determinations was 4% and the inter-assay CV was 10%. The cut off for antibodies to this LV-1-polyprotein (LV-1A) was set to 56 U/ml, which corresponded to the 75th percentile of results of the controls' LV-1A.

Suspension multiplex immunoassay.

Synthetic peptides representing HPeV, LV, Aichi virus and poliovirus (Table I) were used for detecting IgM and IgG antibodies in an exploratory suspension immunoassay [Blomberg et al., 2012]. The *Aichi virus* species in the *Kobuvirus* genus and the *Poliovirus* subtype of the *Enterovirus C* species from the *Enterovirus* genus both belong to the *Picornaviridae* family.

The synthetic peptides were selected from a larger set of peptides containing predicted epitopes [Kolaskar and Tongaonkar, 1990; Larsen et al., 2006] in the Immune Epitope Database (IEDB, <http://tools.immuneepitope.org>). Frequent and strongly reactive peptides in 70 blood donor sera were chosen. All peptides were 30-mers and had a triethylene glycol spacer coupled at the NH₂-terminal end [Elfaitouri et al., 2013]. Coupling of proteins and peptides to carboxylated microspheres was performed as described [Blomberg et al., 2012]. *Haemophilus influenzae* type B vaccine, bovine fetuin for fetuin-IgM and dialyzed inactivated Polio Vaccine (IPV, Imovax® Polio; Sanofi Pasteur MSD, Lyon, France) were used as positive controls and coupled according to the protocol above [Blomberg et al., 2012]. Unprocessed (“naked”) Luminex beads without any bound antigen, were used to control for nonspecific binding as described previously [Elfaitouri et al., 2013]. The exploratory suspension multiplex immunoassay for IgG and IgM required only 5-10 µl of serum or plasma. IgG was detected using biotinylated protein G [Bjorck and Blomberg, 1987] and IgM using biotinylated anti-human IgM (affinity purified µ-chain specific, Sigma-Aldrich, St Louis, MO, USA) as secondary antibody. Filter plate loading and washing for an IgG was performed as described [Sheikholvaezin et al., 2011; Blomberg et al., 2012; Elfaitouri et al., 2013].

Influenza A/H1N1 antibody assay. *InfluenzaA/H1N1pdm2009*, was used as a positive control in the RBA as the pandemic and mass vaccination coincided with the collection period

of the samples. *InfluenzaA/H1N1pdm2009* antibodies were analyzed in 2.5 µl serum in a radioligand binding assay as described in detail [Svensson et al., 2014].

Islet autoantibodies. Autoantibodies against glutamic acid decarboxylase, islet antigen-2, insulin and to Zn-T8 variants (Zn-T8-Arginine, Zn-T8-Tryptophan and Zn-T8-Glutamine) were analyzed at the time of diagnosis as described [Delli et al., 2012].

HLA genotyping. HLA-DQB1 and -DQA1 alleles were typed by sequence-specific oligonucleotide probes using a DELFIA Hybridization assay (Perkin Elmer, Boston, MA, USA) as described [Hagopian et al., 2011].

Statistics

Statistical analyses were performed using SPSS statistical software (version 22[®], SPSS, IBM, Chicago, Illinois, USA). Differences in proportions between groups were tested using the chi-square and two-tailed Fischer's exact test and correlations based on absolute values by Spearman's rho. One-way ANOVA analyses were used for comparing antibody levels between groups. *p*-values <0.05 were considered significant. LV antibody distribution in RBA was evaluated using Quantile-Quantile normal probability plots. A departure from a linear correlation between LV antibody-levels and quantiles of a standard normal distribution would indicate a deviation from normal distribution.

Cut off for positivity was defined at the 99th percentile of controls for the islet autoantibodies. For LV antibodies the cut off was at the 75th percentile of controls. Correlations were based on absolute values (Spearman) and crosstabulation on the 75th percentile of controls.

The results in the suspension multiplex immunoassay were analyzed in the MultiPlus[™] computer program, which subtracts the no-template control (NTC) and naked bead values and checks the results of control sera for each peptide and protein to store them in a relational Visual Foxpro database. The subtraction was trivial for nearly all naked bead values, which were 5-10 mean fluorescence intensity (MFI), but nontrivial for a few peptides, where the NTC values could reach 40-50. In these cases the lowest MFI obtained with some samples were often lower than the NTC value. To avoid over-subtraction, the subtraction was made with 10% of the NTC and 90% of the lowest sample value for the bead. Negative values after subtraction were set to 0 MFI. A cut off at 150 MFI was set for positive values for evaluation of significant differences in antigen reactivity.

When comparing levels of suspension multiplex immunoassay IgG with RBA LV antibody levels a cut off level was also set at 75% of controls antibody levels.

Results

IgM and IgG antibodies to LV-peptides.

Levels of LV-IgM detected in exploratory suspension multiplex assay showed inter-peptide antibody correlation ($p<0.001$) as did LV-IgG for all five LV-peptides ($p<0.001$ for all combinations, Table II). LV-IgM and LV-IgG correlated in 32% of the possible combinations ($p=0.02 - p<0.001$).

LV-IgM above cut off at 150 MFI was found in 15% to LV-polyprotein_2066-2095, in 87% to LV-polyprotein_2193-2222, and in 45% to LV-VP1_568-597 (Figure 1). The cut off level was reached by 0.3% for antibodies to LV-VP1_31-60 but in none of the samples for the LV-polyprotein_1799-1828.

LV-IgG reached the cut off level of 150 MFI in 0-2% of the samples (Figure 2).

Frequency of insulin autoantibodies correlated to LV-VP1_31-60-IgG ($p=0.011$) but not to polyprotein_2193-2222 ($p=0.624$) (data not shown). The LV-VP1_31-60-IgG correlated with insulin autoantibodies alone ($p=0.011$; data not shown) and these autoantibodies in combination with HLA-DQ8 (OR 2.74; 95th CI 1.226-6.143; $p=0.022$) as well as with HLA-DQ 8/8 and 8/X patients (OR 6.07; 95th CI 1.427-25.798, $p=0.013$).

RBA detected LV-1 antibodies

The normal probability plot indicated higher levels of LV-1 antibodies detected by the radio-binding assay at the 4th quartile of serum samples from both controls and type 1 diabetes patients (data not shown). The RBA LV-1 antibody levels in the samples from patients (45.0; range 1-1972 U/ml) were higher than in the controls (27.0; range 3-1488 U/ml), $p=0.24$. The levels in the patients were highest at <5 years of age (55% in 4th quartile, mean 171; range 8-850 U/ml) and 5-9.99 years (67% in 4th quartile, mean 183; range 1918-1972 U/ml) as

compared to the children being 10-14.99 (19% in 4th quartile, mean 62; 1-492 U/ml) and 15-18 (17% in 4th quartile, mean 27; 8-69 U/ml) years of age, $p=0.003$.

The frequency of LV-1 antibodies > 75th percentile in the type 1 diabetes patients was 39% (27/69) and 25% (73/295) in the controls ($p=0.02$). The frequency was higher in type 1 diabetes children with age at diagnosis below (62%) compared to older than 10 years of age (17%; $p<0.001$). The frequency of LV-1 antibodies > 75th percentile was compared to patients autoantibodies to insulin (33%), glutamic acid decarboxylase (41%), islet antigen-2 (90%) and to Zinc transporter variants; Zn-T8-Arginine, (67%), Zn-T8-Tryptophane (57%) and Zn-T8-Glutamine (49%). LV-1 antibodies correlated to insulin autoantibody levels in the type 1 diabetes patients ($r_s=0.319$; $p=0.008$) while there was no correlation to the levels of the other islet autoantibodies.

The frequency of RBA LV-1 antibodies > 75th percentile was associated with HLA-DQ8 in patients and controls together ($p=0.024$, Table III). There was an association between LV-1 antibodies and insulin autoantibodies in patients with HLA-DQ 8/8 and 8/X ($p=0.014$, Figure 3). For patients and controls together see Table IV. LV-1 antibodies were related to IgG against LV-peptide-VP1_31-60 ($p=0.009$, Table II).

IgM and IgG antibodies to HPeV3-peptides

Levels of HPeV3-IgM and HPeV3-IgG showed inter-peptide correlations ($p<0.001$) for all but two combinations of IgM and significant for 83% of all IgG combinations. Inter-peptide correlations between IgM and IgG to HPeV3 were statistically significant in 31% of all possible combinations (data not shown).

IgM antibody frequency above the cut off at 150 MFI was found in 93% for HPeV3-VP1_171-200 (median 513.96, range 5.46– 2924.46 MFI) and 88% for HPeV3-VP1_176-205 (median 296.71, range 10.71– 2294.71 MFI, Figure 1). These two peptides had 23 consecutive identical amino acids. The frequency of IgM antibodies above cut off varied

between 14 – 27% for HPeV3-VP1_1-36, VP1_37-66, VP1_43-77 and VP1_95-124 while for the rest of the HPeV3-peptides the frequency was 1-6%.

IgG antibody frequency above cut off at 150 MFI was found for three of the ten HPeV3-peptides used in the analyses (Figure 2), of which two of them had identical sequences for 23/30 amino acids (Table I).

IgG levels were higher in type 1 diabetes patients than in controls for HPeV3-VP1_43-72, ($p=0.007$), HPeV3-VP1_64-93 ($p<0.001$), HPeV3-VP1_124-153 ($p=0.04$), HPeV5-VP1_31-60 ($p<0.001$) and HPeV2-polyprotein_1992-2021 ($p=0.032$).

IgG to HPeV3-VP1_1-30 ($p<0.001$) and to VP1_95-124 ($p=0.009$) but none of the other HPeV3-IgG were related to the LV-1 antibodies (Table IV). However, IgG to HPeV3-VP1_1-30 ($p=0.072$) or VP1_95-124 ($p=0.254$) did not correlate to insulin autoantibodies. There was no interrelationship between IgG to HPeV3-VP1_1-30, insulin autoantibodies and HLA-DQ (OR=2.18; 95th CI 0.975-4.892, $p=0.072$), except in subjects with HLA-DQ 8/8 and 8/X (OR=10.40; 95th CI 2.031-53.243, $p=0.002$).

IgM and IgG antibodies to HPeV2, 5, 6 and 7 –peptides

The inter-peptide correlations for antibodies to HPeV2, 5, 6 and 7 were significant for both IgM ($p<0.001$) and IgG ($p<0.001$) except for both IgM and IgG against the HPeV2-polyprotein_1726-1755. The inter-IgM-IgG correlations for these peptides were significant in 22% of the combinations (data not shown).

IgM antibody frequency above the cut off at 150 MFI was found in 77-99.7% for the HPeV2-polyprotein_1992-2021, HPeV2-polyprotein_2118-2147 and HPeV5-VP1_31-60 (Figure 1). IgM frequency above cut off was 20% for HPeV7_46-75, 9% for HPeV2-polyprotein_1726-1755 and 0% for HPeV5_1-30 as well as for HPeV6_51-80. IgG antibody levels above cut off at 150 MFI was found in 0 – 0.6% (Figure 2).

Among the HPeV2, 5, 6, 7 peptides there was a significant correlation between LV-1 antibodies and IgG to HPeV6_51-80 (which had 52% zero values and no value above >150 MFI) in type 1 diabetes patients alone ($p=0.05$) as well as patients and controls combined. Similarly, in patients and controls together, LV-1 antibodies correlated to HPeV5_31-60-IgG, which had measurable values in 16% of the samples but no value above 150 MFI.

IgM and IgG antibodies to Aichi virus and poliovirus

IgM and IgG against the one Aichi virus peptide tested correlated ($p=0.02$). Aichi virus above the 150 MFI cut off were found for IgM in 19% and for IgG in 0.3% of the samples.

IgM-antibodies to poliovirus peptides VP1_2150-2179 and VP1_2021-2050 above the 150 MFI cut off level were found in 100% of the samples compared to 3% for polio vaccine (Figure 1). The frequency of poliovirus-VP1_1769-1798 IgM-antibodies above cut off was 0.8%. IgG-antibodies to poliovirus were detected at levels above the 150 MFI cut off at a frequency of 80% for IgG to polio vaccine, 94% to poliovirus VP1_2021-2050, in 2% to VP1_2150-2179 and in 1% to VP1_1769-1798 (Figure 2).

Neither IgM nor IgG to Aichi virus peptide, showed any correlation to the LV-1 antibodies.

Hemophilus influenzae B vaccine antibodies

Hemophilus influenzae B IgG antibodies serving as positive control for the assay were detected in all samples and 98% of the samples had levels above the 150 MFI cut off.

RBA A/H1N1pdm2009 antibodies

The normal probability plots for A/H1N1 antibodies in controls and type 1 diabetes patients showed increased levels in the 4th quartile. The influenzaA/H1N1 pandemic and mass vaccination period coincided with sampling time, however, only nine type 1 diabetes patients were diagnosed during this period. Over 80% of the children that left blood samples during,

and after the pandemic and mass vaccination were vaccinated against *A/H1N1pdm2009* (source: CDC, County of Jämtland, Sweden). InfluenzaA/H1N1 antibody levels showed significant increase comparing before (n=76, mean 18, range 1-125 U/ml) to during October 2009 - March 2010 (n=98, mean 95, range 2-1640 U/ml; $p=0.028$ compared to before), and after (n=189, mean=101, range 2-1587 U/ml; $p<0.001$ compared to before) the influenzaA/H1N1 pandemic and mass vaccination. There was no significant difference in levels of the other antibodies tested comparing before, during and after influenzaA/H1N1 pandemic and mass vaccination (data not shown).

Discussion

The patients and controls in the present study represent a second not previously studied cohort of type 1 diabetes patients and controls from the county of Jämtland in Sweden. In the first study patients and controls from this county were found to show an overall distribution of antibodies to LV suggesting prior exposure with varying antibody levels in the radio binding assay that primarily detect IgG [Nilsson et al., 2009]. As the first study had a limited number of patients and controls it was followed up by a study of 676 newly diagnosed type 1 diabetes patients in Region Skåne (most southern region of Sweden) ascertained over 10 years [Nilsson et al., 2013]. LV antibody levels and frequencies were comparable to the first study in Jämtland suggesting that a significant proportion of subjects, type 1 diabetes patients as well as controls, had been exposed to LV or a cross-reactive virus. Furthermore, the Skåne study suggested that LV antibodies were related to young age at onset, insulin autoantibodies and HLA-DQ8 [Nilsson et al., 2013].

The present study is a newly ascertained cohort of Jämtland patients and controls. The major finding of this study is that LV-1 antibodies proved related to young age at onset, insulin autoantibodies and HLA-DQ8. This relationship was significant despite the present patient population was only one tenth of that of the Skåne study [Nilsson et al., 2013]. There is no sequence homology between insulin and the LV polyprotein used in the LV RBA i.e. there is no support for molecular mimicry.

The second major finding in the present study was that the IgG antibodies detected against virus peptides in the suspension multiplex immunoassay correlated between each of the different LV peptides and also to LV-1 antibodies detected by the RBA for three of them; LV-IgG against VP1_31-60 and to LV-polyprotein_2193-2222 and LV-polyprotein_1799-1828. The observation that levels of RBA detected LV-1 antibodies did not correlate to any of the

LV-IgM but exclusively to LV-IgG peptide antibodies would be consistent with the findings that RBA detected LV-1 antibodies primarily represents IgG1, IgG2 or IgG4 subtypes known to react with Protein A. Sepharose-protein A is used in the RBA to separate antibody-bound from free labeled antigen [Grubin et al., 1994]. The fact that the suspension immunoassay LV-peptide and LV-1 antibodies did correlate with peptides also represented in the LV cDNA used in the *in vitro* transcription translation is an indication that some children have been exposed to either LV or perhaps a closely related parechovirus. The time-point of exposure was not possible to determine in the present set of samples ascertained in this cross-sectional manner. The quantile-quantile normal probability plots of binding levels may be interpreted as if the very high levels may represent more recent exposure while the lower levels may represent exposures more distant in time. The explanation would be that levels decrease over time when the antigen has been neutralized and is no longer present. This interpretation is consistent with the recent report that LV antibodies by indirect immunofluorescence showed that 38% of the Finnish population were likely to have been exposed [Jaaskelainen et al., 2013].

It is a significant finding in the present analyses that multiplex suspension immunoassay HPeV-peptide antibodies also occurred more often in subjects positive for insulin autoantibodies and HLA-DQ8. None of the multiplex suspension immunoassay peptide antibodies, be it IgM or IgG, showed any correlation to any islet autoantibody except for the insulin autoantibodies.

It was questioned whether the RBA for LV-1 antibodies might show cross-reactivity with related viruses indicating that the antibodies detected would not be specific but rather represent HPeV antibodies that are able to immunoprecipitate the *in vitro* transcription translation product of the LV cDNA used in the present study. A large panel of HPeV peptides representing different parts of the viral genome was therefore prepared. The IgM and

IgG analyses here did not provide strong evidence for that HPeV antibodies would solely cross-react with the LV translation product. The finding that levels of RBA LV-1 antibodies correlated with IgG to HPeV3-VP1_1-30 and -VP1_95-124 is more likely to be explained by simultaneous exposure as neither one of these two peptide IgG were related to insulin autoantibodies or HLA-DQ8. One interpretation is that it is not uncommon that subjects may have been exposed to both LV and another parechovirus. This interpretation is consistent with recent reports that a novel porcine parechovirus-like virus primarily infect young children [Yu et al., 2013] and that LV and parechoviruses are common in rodents in the UK [Salisbury et al., 2013]. Other studies suggest that parechovirus exposure in adult humans are exceedingly common [Joki-Korpela and Hyypia, 2001] and that it therefore cannot be excluded that some suspension multiplex immunoassay detected IgG against HPeV i.e. IgG-LV-VP1_31-60 may occur at the same time as antibodies RBA detected against LV-1, but that it is not the same antibodies that would recognize the different antigens. Cross-absorption studies may shed light on the matter, but serology alone cannot support a final conclusion. Neutralization assay testing the capacity of serum to neutralize the activity of a virus would give a more conclusive result.

The present set of 26 different peptides of various virus allowed us extensively to analyze the specificity in antibody reactivity to different virus. A strength of our observations was that the interrelationship in IgM and IgG binding to individual peptides was strong. The pattern of reactivity to different parts of the virus antigen is interpreted as a highly polyclonal reaction to a prior virus infection. The absence of an inter-relationship between peptide IgM and IgG to different viruses further suggest that the immune response is highly specific. Further analysis of the seroprevalence of these antibodies in the general population will be needed to determine whether the immune response is associated with HLA-DR-DQ haplotypes similar to what we now report for LV-1 antibodies and IgG-LV-VP1_31-60 with HLA-DQ8. It is of

interest in this regard that there is a relationship between HLA-DR3-DQ2 and enterovirus IgM in pregnant women and risk for islet autoantibodies during the first trimester [Resic Lindehammer et al., 2012].

As the time for sample collection coincided with the epidemic and vaccination period of influenzaA/H1N1, the “new influenza” or Swine flu [Bardage et al., 2011; Svensson et al., 2014] analyses were performed for antibodies against influenzaA/H1N1 by a RBA as well. Many, but not all, of the children in the control group were vaccinated against influenzaA/H1N1 before blood sampling. The increasing frequency of antibodies to influenzaA/H1N1 in the samples collected from autumn of 2009 onwards would be consistent with the pandemic and mass vaccination period.

In conclusion, the present study in type 1 diabetes patients and controls ascertained in Jämtland county in Northern Sweden suggests that these individuals have been exposed to LV, measured as both IgG antibodies against LV-1 and suspension multiplex immunoassay. Possible antibody cross-reactivity of LV and HPeV cannot be excluded. However, the study supports previous observations that LV but not HPeV antibodies were related to type 1 diabetes as LV antibody titers were related to insulin autoantibodies, but not to other autoantibodies. Most stringent associations were found in young age-at-onset type 1 diabetes children and positive for HLA-DQ8. IgM and IgG antibodies against poliovirus, influenza or Haemophilus B did not show any relationship to type 1 diabetes or HLA-DQ. These data further support the notion that exposure to LV, or a closely related virus at a young age, may be related to the risk of type 1 diabetes.

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Tables:

Table I. Amino acid sequence of peptides used in the suspension multiplex indirect immunofluorescence assay to detect IgM and IgG against peptides selected from human parechovirus (HPeV), Ljungan virus, Aichi virus and poliovirus.

Peptide name and sequence
HPeV 3 VP1 1-30 MENCKQSISPNELGLTSAQDDGPLGNEKPN
HPeV 3 VP1 37-66 TMNVDIFTVSHTKVDNIFGRAWYVTSHDFN
HPeV3 VP1 43-72 FTVSHTKVDNIFGRAWYVTSHDFNNGDTWR
HPeV3 ACT09063 VP1 46-75 KPNYFLNFRTMNVDIFTVSHTKVDNIFGRA
HPeV3 VP1 64-93 DFNNGDTWRQKLTFPKEGHGMLSQFFAYFT
HPeV3 VP1 95-124 EINIHLIYMAEQGFLRVAHTYDTEENRKT
HPeV3 VP1 124-153 FLSSNGVITIPAGEQMTLSVPFYSNKPLRT
HPeV3 VP1 139-168 MTLSVPFYSNKPLRTVRHDSALGFLMCRPM
HPeV3 VP1 171-200 GTTRTTAEVYISLRCPNFFFPVPAPKPTGS
HPeV3 VP1 176-205 TAEVYISLRCPNFFFPVPAPKPTGSGAAAL
HPeV7 VP1 46-75 AFV67943 KPNYFLNFRAMNVDIFTASHTKVDNIFGRA
Ljungan virus NP_705876 VP1_31-60 NVEAAQGEEAAATEVGLRATENDGSLSEQLN
Ljungan virus ACJ48052 VP1_568-597 AGPMFLNYKQHNVDIFSASHTKVDHIFGRA
Polio1 VP1 1769-1798 SKTKLEPSAFHYVFEGVKEPAVLTKNDPRL
Polio1 VP1 2150-2179 RWTGDPRNTQDHVRSLLAWHNGEEYNK
Polio1 VP1 2021-2050 HLYKNKTYCVKGGMPSGCSGTSIFNSMINN
HPeV2_polyprot_CAA06679 1726-1755 TKSQIHKSPVYGAVEVKMGPAVLKSDTRL
HPeV2_polyprot_CAA06679 1992-2021 HVVFNERWLIHGGMPSGSPCTTVLNSLCNL
HPeV2_polyprot_CAA06679 2118-2147 MWMKNFSTFKQQLQSYLMELCLHGKDTYQH
HPeV 5 1-30 GDEAEDCKQTISPDELGLTSAQDDGPLGME
HPeV 5 31-60 KPNYFLNFRAINVDIFTVSHTKVDNIFGRA
HPeV6 ABZ80978 51-80 EDDAENCKQTISPNELGLTSAQDDGPLG
Ljungan virus NP_647602 polyprot 1799-1828 SKTALNPSPNLGVVPVKMEPAVLSPHDTRL
Ljungan virus NP_647602 polyprot 2066-2095 HLVMDEIWHVNGGMPSGAPCTTVLNSICNL
Ljungan virus_AA083985 polyprot_2193-2222MWMKNLSTFPEQLVSFENELALHGKDVYDD
Aichi virus 3D NP_859028.1 17-46 RKSQGLGHSPAYGAFPVKKQPAVLHQRDARL

Table II. Correlations between Ljungan virus antibodies (LV-1A) detected in the radiobinding assay (RBA) and IgG antibodies to Ljungan virus peptides detected in the multiplex indirect immunofluorescence assay in controls and type 1 diabetes patients, n=363.

IgG antibodies	RBA LV-1A	LV-VP1 _31-60	LV-VP1 _568-597	LV_1799 -1828	LV_2066 -2095	LV_2193 -2222
RBA LV-1A	1.000	0.009	0.065	0.022	0.081	0.001
LV-VP1_31-60	0.136	1.000	0.000	0.000	0.000	0.000
LV-VP1_568-597	0.097	0.233	1.000	0.000	0.000	0.000
LV_1799-1828	0.120	0.488	0.242	1.000	0.000	0.000
LV_2066-2095	0.092	0.465	0.270	0.289	1.000	0.000
LV_2193-2222	0.182	0.315	0.191	0.240	0.335	1.000

The table indicates correlation coefficients below and *p*-values above the line of unity. Peptide abbreviations are shown in Table I.

Table III. Association between HLA-DQ genotypes and Ljungan virus antibody (LV-1A) frequency, radiobinding assay, in T1D patients and controls. Cut off level at 75% of controls LV-1A.

HLA-DQ	LV-1A	Controls (n)	T1D (n)	Total	<i>p</i> -value
2/8	neg	5	13	18	0.677
	pos	2	9	11	
8/8,8/ <i>X</i>	neg	44	21	65	0.024
	pos	6	11	17	
2/2,2/ <i>X</i>	neg	42	3	45	0.182
	pos	15	4	19	
<i>X</i> / <i>X</i>	neg	130	5	135	0.689
	pos	50	3	53	
Total	neg	221	42	263	0.024
	pos	73	27	100	

p-value in two-sided Fischer's exact test.

Table IV. Association between HLA-DQ genotypes, insulin autoantibodies (IAA) and Ljungan virus antibodies (LV-1A), radiobinding assay, frequency in T1D patients and controls together.

HLA-DQ	n	LV-1A	IAA		Total	<i>p</i> -value
			Neg.	Pos.		
2/8	29	neg	12	6	18	1.000
		pos	8	3	11	
8/8, 8/X	82	neg	61	4	65	0.004
		pos	11	6	17	
2/2, 2/X	64	neg	44	1	45	0.208
		pos	17	2	19	
X/X	188	neg	133	2	135	0.316
		pos	51	2	53	
Total	363	neg	250	13	263	0.012
		pos	87	13	100	

p-value in two-sided Fischer's exact test.)

Table V. Correlations between Ljungan virus IgG antibodies (LV-1A) detected in the radiobinding assay (RBA) and IgG antibodies to human parechovirus 3, HPeV3, peptides detected in the multiplex indirect immunofluorescence assay in controls and type 1 diabetes patients, n=363.

*HPeV3	LV-1A	*VP1_ 1-30	*VP1_ 64-93	*VP1_ 95-124	*VP1_ 171-200	*VP1_ 176-205
LV-1A	1.000	0.000	0.443	0.009	0.349	0.236
*VP1_1-30	0.264	1.000	0.000	0.000	0.003	0.008
*VP1_64-93	0.040	0.257	1.000	0.000	0.000	0.001
*VP1_95-124	0.138	0.282	0.398	1.000	0.000	0.000
*VP1_171-200	0.049	0.158	0.190	0.000	1.000	0.000
*VP1_176-205	0.062	0.139	0.176	0.227	0.839	1.000

The table indicates correlation coefficients below and *p*-values above the line of unity.

Peptide abbreviations are shown in Table I.

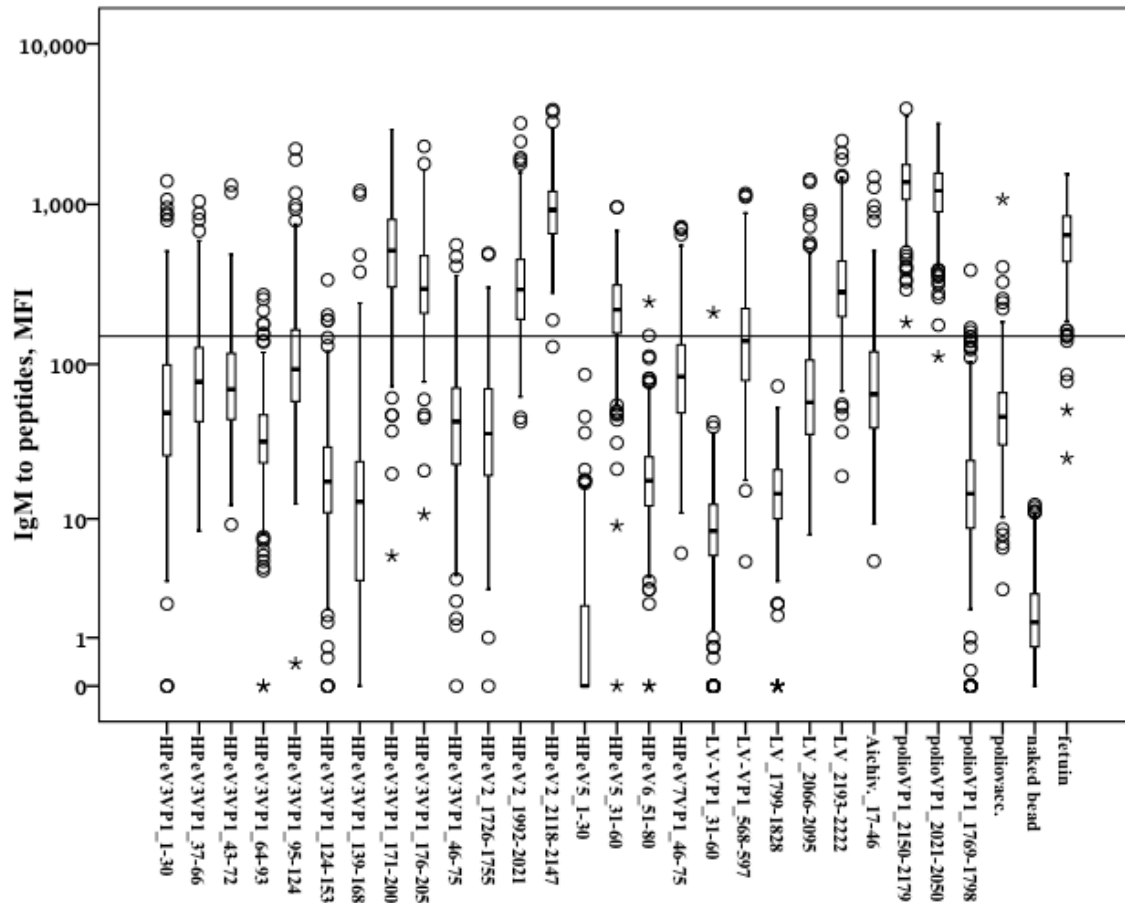


Figure 1. IgM antibodies detected by suspension multiplex immunoassay against peptides from human parechovirus (HPeV strains 2, 3, 5, 6 and 7), Aichi virus, poliovirus, Ljungan virus (LV), and fetuin in 1-18 years old type 1 diabetes patients and controls combined (n=363).

Box plots with whiskers and outliers are shown on a logarithmic scale. Suspension multiplex immunoassay as mean fluorescence intensity (MFI, cut off line at 150).

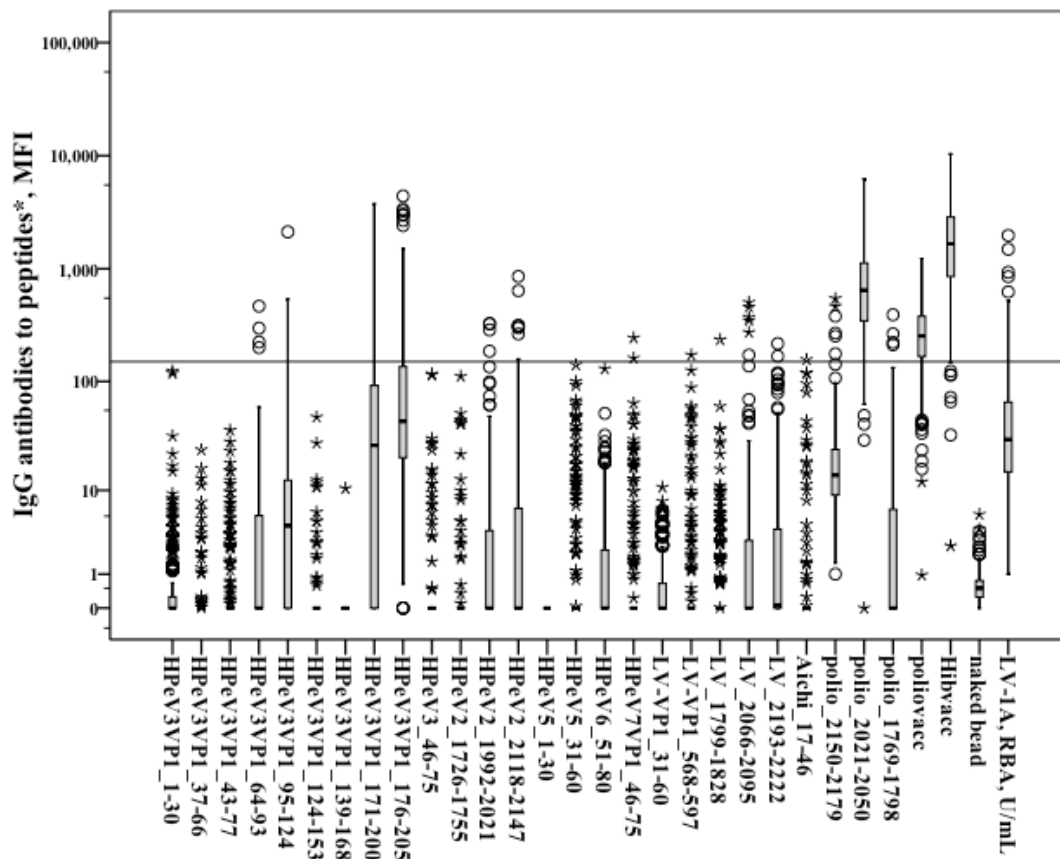


Figure 2. IgG antibodies detected by suspension multiplex immunoassay against peptides from human parechovirus (HPeV strains 2, 3, 5, 6 and 7), Aichi virus, poliovirus, Ljungan virus (LV), and *Hemophilus Influenzae B* in 1-18 years old type 1 diabetes patients and healthy controls combined (n=363). Ljungan virus antibodies (LV-1A, U/mL, mean values) by radiobinding assay (RBA) for comparison.

Box plots with whiskers and outliers are shown on a logarithmic scale. Suspension multiplex immunoassay as mean fluorescence intensity (MFI, cut off line at 150).

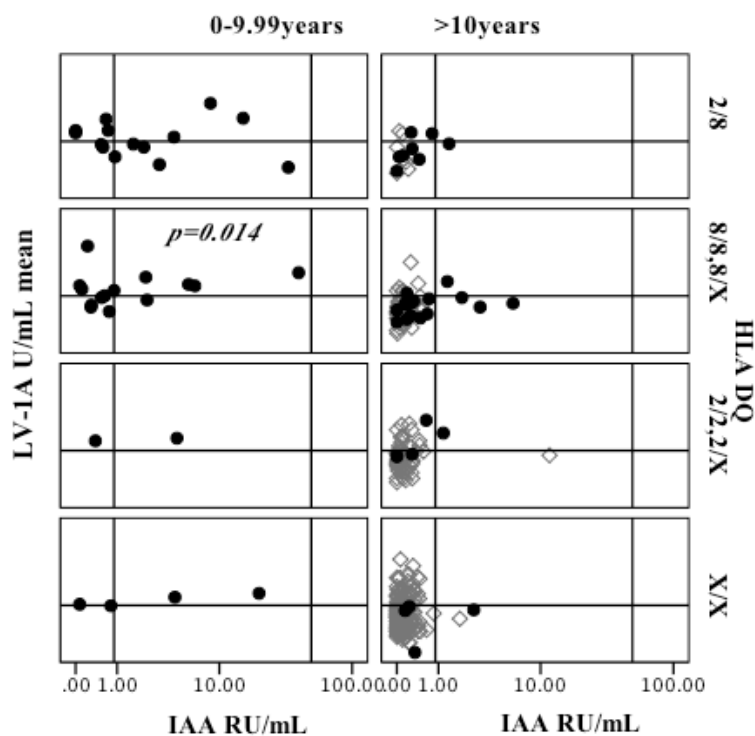


Figure 3. LV-1 antibodies (LV-1A, radiobinding assay) U/ml, insulin autoantibodies (IAA, RU/ml) and HLA-DQ genotype in children below 10 years and 10-18 years in 295 healthy controls (▢) and 72 newly diagnosed T1D patients (●). There was a significant association between LV-1 antibodies and insulin autoantibodies in HLA-DQ 8/8 and 8/X children being younger than 10 years of age, $p=0.014$.

Antibody levels are shown on a logarithmic scale. X=neither 2 nor 8.