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Detection of a low-grade enteroviral infection in the islets of Langerhans of living patients newly diagnosed with type 1 diabetes

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Auto-antibody analyzes

Diabetes-related auto-antibodies (Glutamic acid decarboxylase autoantibodies (anti-GAD), insulin autoantibodies (IAA), autoantibodies targeting the phosphatase-related IA-2 molecule (anti-IA2) and zinc transporter autoantibodies (anti-ZnT8)) where measured using radiobinding assays according to Petersen et al[1] at the Hormone Laboratory, Oslo University Hospital, Aker, Oslo, Norway. This laboratory has participated in the Antibody Standardization Program (DASP) since 2003[2]. The indicators for the GAD, IA2 and anti-ZnT8(R+W)A assays were made from 3H labelled in vitro-transcribed/translated clones. The clones were obtained from Drs. Dyrberg (GAD)[1], Gale, University of Bristol, UK (IA2) and Hutton, University of Colorado, CO, USA (ZXnT8). The indicator for IAA was 125I A-14 labeled insulin (Millipore, Billerica, MA, USA).

Islet isolation

The pancreatic duct was located under a surgical microscope, cannulated with a fine catheter, and collagenase (Liberase®, Roche) was injected continuously. After about 30 min, the pancreatic tissue was cut into 3–4 pieces, transferred to two 15 mL glass tubes and digested at 37°C with continuous shaking for 30 min. Each digested pancreas was washed twice with cold culture media (CMRL-1066, ICN Biomedicals, Costa Mesa, CA supplemented with 10 mM HEPES, 2 mM L-glutamine, 50 µg/mL Gentamycin, 20 µg/mL Ciproxfloxacin, and 10% heat-inactivated fetal calf serum) and the digest immediately transferred to culture dishes. 300-700 islets from each patient were handpicked from the digest tissue under a dissecting microscope.

Virus isolation

Virus isolation was undertaken in Uppsala. Culture medium from purified islets and exocrine cell clusters at different time points post isolation (days 1, 3, and 6) were inoculated onto monolayers of permissive cells in 24 well plates (Costar, Corning Inc., US). Cells lines used for virus isolation were HeLa, RD, GMK and EndoC-βH1 cells. Primary human islets were also cultured and inoculated with culture medium as described above. Three of the cell lines (HeLa, RD and GMK cells) were cultured in EMEM (SVA, Uppsala, Sweden) without any supplementation with calf serum at the time of inoculation. The endoC-βH1 cells were cultured in DMEM containing 5·5 mM glucose supplemented with 2% BSA. Primary human islets were cultured in RPMI 1640 containing 5·5 mM glucose (SVA, Uppsala Sweden) with the addition of 5 % foetal bovine serum. Cells were observed daily for evidence of a visible cytopathic effect and EV PCR was performed on cultures that demonstrated a change in cell morphology.

RNA extraction, RT-PCR and sequence analysis

RNA was extracted for analysis of viral RNA by RT-PCR in two laboratories (Tampere and Uppsala). The Uppsala laboratory extracted viral RNA from islet culture medium recovered from islets isolated from all six cases and six controls, at different time points, using the QIamp UltraSens virus kit (Qiagen). The Tampere laboratory extracted RNA from culture media harvested after islet and exocrine cell culture (cultured in Uppsala), and from snap-frozen pancreas biopsy. The presence of several different viruses was analysed using RT-PCR. Enterovirus and rhinovirus RNA was analysed using two independent methods, a qualitative RT-PCR method[3] and a real-time RT-PCR method[4], both including a probe for the detection of virus specific amplification. In addition parechovirus, norovirus and rotavirus were analysed in pancreas extracts using Quantitect Probe kit according to the manufacturer's instructions. The oligonucleotide sequences and concentrations of primers are described in supplementary table 2. A sample was considered positive if it tested positive in at least one of three parallel tests.

In Tampere, the snap-frozen pancreatic biopsy samples were homogenized using a SilentCrusher S homogenizer (Heidolph, Schwabach, Germany) prior to RNA extraction. RNA was extracted from homogenized biopsies and from PBMC samples with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and from serum, and stool samples (10% stool suspension in RPMI) using Viral RNA kit (Qiagen, Hilden, Germany) as described previously[4-6]. The Uppsala laboratory detected enterovirus RNA using a nested RT-PCR. 50 ng total RNA /sample were primed with virus specific primers and reverse transcribed to cDNA with SuperScriptII™ RT (Invitrogen) and/or Sensiscript RT kit (Qiagen) according to the manufacturer's instructions. A nested EV-PCR was performed in the conserved 5´region of the genome; primers used are described in supplementary table 2. Positive PCR products were excised from the gel and purified using Mini elute gel extraction kit (Qiagen, Sweden) according to the manufacturer's instructions. The eluted DNA concentration was determined and the products were sequenced at the Uppsala Genome Center (Uppsala, Sweden) with the same primers as had been used to amplify the 5'UTR region. Nucleotide sequences were identified in the Genebank database by using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

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Sequencing of virus positive samples
Enterovirus positive PCRs from culture media and biopsies were sequenced at the core facility at Uppsala University. Primer sequences are shown in Supplementary Table 2.

High-throughput RNA-sequencing
In Oslo, total RNA from frozen (-80C) whole tissue from patients 1-6 was isolated using the RNeasy Plus Mini Kit, with gDNA eliminator columns and on-column DNAseI digestion (Qiagen, Hilden, Germany). Approximately 30 mg tissue was homogenized in lysis buffer using an Omni Tip homogenizer (Omni International, Kennesaw, GA, USA) with disposable tips. RNA quality and quantity were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and NanoDrop 2000 Spetrophotometer (NanoDrop products, Wilmington, DE, USA). Quality of the RNA isolates was sufficient for sequencing, with RNA integrity valued (RIN) 7·1-9·5 and A260/230 and A260/280 ratios of 2·0 or better (Supplementary Table 4).

1-2 µg total RNA from each sample was treated with the Ribo-Zero Magnetic rRNA Removal Kit (Human/Mouse/Rat) or Ribo-Zero Magnetic Gold Kit (Epicentre Biotechnologies, Madison, WI, USA) and prepared for 100 bp paired-end sequencing and barcoded using the TruSeq RNA Sample Preparation Kit (Illumina) without the mRNA selection step. Sequencing was done on a HiSeq 2000 instrument (Illumina) at the Norwegian Sequencing Centre (Oslo, Norway). Libraries from sample 2 and 6 were spiked with approximately 1% of the PhiX control library according to Illumina recommendations.

Analysis of RNA-sequencing results
Raw sequences were generated and demultiplexed using RTA and CASAVA (Illumina), and the quality of sequences were evaluated using FastQC v0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Estimates using 100k random single reads from each sample with FastQ screen v0·4 (http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/) showed that the rRNA content in generated sequences ranged from approximately 4-47%, while the total number of reads not mapping to human RNA or DNA ranged from 6-12%. Sequences were filtered for low quality reads and sequencing artefacts with the FastX toolkit v0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/), using a minimum threshold of 80% nucleotides with PHRED=20. The resulting number of paired-end reads ranged from 71-119M (Supplementary Table 4).

RNA-sequencing data was searched for viral sequences using the Rapid identification of non-human sequences (RINS) software[7], modified to use the Trinity RNA-Seq de novo assembler release 2012-03-17[8], and using the virus database accompanying the RINS software. After cleaning (duplicates and non-viral sequences removed) this database contained 28864 viral reference sequences.

Sensitivity of RNA-sequencing
Testing random downsampling of reads from the two samples spiked with PhiX (cases 2 and 6), as few as 5-8 raw paired-end reads falling on this 5386 nt template are needed for positive detection using the RINS method. With the 86-94M paired-end reads generated for sample 2 and 6, the detection limit of viral RNA reads should be as low as 1:17M. This is however only an approximation, and will particularly depend on the length of the virus genome. Note also that DNA viruses in the original samples are not detected by our method unless there is also transcriptional activity.

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Supplementary Table 1. Demographic data, controls.

Control (nPOD-number)	Age	Sex	BMI	Anti GAD	Anti-insulin	anti-ZnT8	Anti IA2	HLA
nPOD 1 (6001)	22	M	21,9	Negative	Negative	Negative	Negative	A*02/02, B*07/62, DR*04/15, DQ*/
nPOD 2 (6024)	21	M	27,8	Negative	Negative	Negative	Negative	A*01/31, B*07/08, DR*13/17, DQ*/
nPOD 3 (6030)	30,1	M	27,1	Negative	Negative	Negative	Negative	A*02/31, B*07/44, DR*15/07, DQ*06/02
nPOD 4 (6034)	32	F	25,2	Negative	Negative	Negative	Negative	A*03/03, B*07/62, DR*01/08, DQ*05/04
nPOD 5 (6073)	19,2	M	36,0	Negative	Negative	Negative	Negative	A*29/01, B*37/44, DR*13/16, DQ*05/06
nPOD 6 (6098)	17,8	M	22,8	Negative	Negative	Negative	Negative	A*01/32, B*08/27, DR*17/08, DQ*02/04
nPOD 7 (6140)	38	M	21,7	Negative	Negative	Negative	Negative	A*01/03, B*35/35, DR*01/17, DQ*05/02
nPOD 8 (6160)	22,1	M	23,9	Negative	Negative	Negative	Negative	A*11/29, B*35/44, DR*103/07, DQ*02/05
nPOD 9 (6178)	24,5	F	27,5	Negative	Negative	Negative	Negative	A*02/24, B*27/44, DR*04/15, DQ*06/03
Uppsala1	54	F	25.7	Negative	Not tested	Not tested	Negative	A*02/03, B*35/35, DR*01/01, DQ*05/05
Uppsala2	64	M	24.7	Negative	Not tested	Not tested	Negative	A*01/24, B*27/27, DR*01/01
Uppsala3	69	F	19.5	Negative	Not tested	Not tested	Negative	A*02/26, B*62/64, DR*04/16
Uppsala4	68	F	25.3	Negative	Not tested	Not tested	Negative	A*01/02, B*08/40, DR*03/12, DQ*02/03
Uppsala5	69	M	25.7	Negative	Not tested	Not tested	Negative	Not available
Uppsala6	64	M	28.9	Negative	Not tested	Not tested	Negative	A*01/02, B*08/62, DR*04/17, DQ*02/03

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Supplementary Table 2. Methods of immunohistochemistry.

Primary Antibody	Manufacturer and clone	Laboratory	Staining procedure (automated or manual)	Antigen Retrieval	Antibody Dilution	Incubation time with primary antibody	Secondary Detection System
VP1	Dako 5D8/1	Exeter, UK	Manual	10mM citrate pH6·0	1/2000 (55ng/ml)	1h at RT	Dako REAL™ Envision™ Detection System
		Tampere, Finland	Automated (Ventana BenchMark LT autostainer)	Tris-EDTA pH 8·5	1:300	30 min at 37°C	*ultra*View™ Universal detection system (Ventana Medical Systems)
Class I MHC	Abcam EMR8-5	Exeter	Manual	10mM citrate pH6·0	1/1500	1h at RT	Dako REAL™ Envision™ Detection System
Insulin	Dako C#A056401	Exeter Combination Stain	Manual	10mM citrate pH6·0	1/600	1h at RT	Dako REAL™ Envision™ Detection System
Glucagon	Abcam C#ab82270				1/2000	1h at RT	Vector AP-ABC kit combined with Vector Red Substrate kit

Supplementary Table 3. PCR-primers and probes

Virus	Primer name	Sequence	Concentration (µM)
Enterovirus	fwd 636	CGGCCCCTGAATGCGGCTAA	**900**
	rev 4-	GAAACACGGACACCCAAAGTA	**900**
Rhinovirus	fwd 636	CGGCCCCTGAATGCGGCTAA	**900**
	rev 4-	GAAACACGGACACCCAAAGTA	**900**
Norovirus	NoroG2 fwdQ	CARGARBCNATGTTYAGRTGGATGAG	**900**
	NoroG2 revQ	TCGACGCCATCTTCATTCACA	**300**
	NoroG1 revQ:	CTTAGACGCCATCATCATTYAC	**900**
	NoroG1 fwdQ	CGYTGGATGCGNTTYCATGA	**900**
Rotavirus	VP2-F1	TCTGCAGACAGTTGAACCTATTAA	**900**
	VP2-F2	CAGACACGGTTGAACCCATTAA	**900**
	VP2-F3	TCGGCTGATACAGTAGAACCTATAAATG	**900**
	VP2-F4	TGTCAGCTGATACAGTAGAACCTATAAATG	**900**
	VP2-F5	TCAGCTGACACAGTAGAACCTATA AATG	**900**
	VP2-R1	GTTGGCGTTTACAGTTCGTTCAT	**50**
	VP2-R2	GTTGGCGTCTACAATTCGTTCAT	**50**
Parechovirus	ParE AN345	GTAACASWWGCCTCTGGGSCCAAAAG	**300**
	ParE AN344	GGCCCCWGRTCAGATCCAYAGT	**300**
Enterovirus	FW	GCCCCTGAATGCGGCTAAT	**100 pmol/µl**
	Rev	GATGGCCAATCCAATAGCT	**100 pmol/µl**
	Rev	ATTGTCACCATAAGCAGCCA	**100 pmol/µl**
Virus	**Probe**	**Sequence and label**	
Enterovirus	Q-PCREVI	FAM-TCTGTGGCGGAACCGACTA-TAMRA	**300**
	Q-PCREVII	FAM-TCTGCAGCGGAACCGACTA-TAMRA	**300**
Rhinovirus	Rhinoprobe	VIC- CGGGAIGGGACCAACTA -TAMRA	**300**
Norovirus	NoroG2probeQ	FAM-TGG GAG GGC GAT CGC AAT CT-TAMRA	**300**
	NoroG1probeQ	VIC-AGA TYG CGA TCY CCT GTC CA- TAMRA	**250**
Rotavirus	RotaVp2-P	FAM-ATG CGC ATR TTR TCA AAH GCA A-MGB-NFQ	**200**
Parechovirus	ParE AN257	FAM-CCTRYGGGTACCTYCWGGGCATCCTTC-TAMRA	**200**

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Supplementary Table 4. Sequence alignment of PCR-products, Uppsala. Variable nucleotide sites between different cases are highlighted with yellow.

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Case2  GTACCTTTGTGCGCCTGTTTTATTACCCCCTCCCCTAACTGTAACTTAGAAGTAATACACACCGATCAACAGTAAGTGTGGCACGCCAGCCATGCTCCGATCAAGCACTTCTGTTCCCCC
Case4  ----------------------------------------------------------------------------------------------------------------------------
Case5  ----------------------------------------------------------------------------------------------------------------------------
Case6  ----------------------------------------------------------------------------------------------------------------------------

Case2  GGACCGAGTATCAATAGACTGCCCACGCGGTTGAAGGAGAAAGTGTCCGTTATCCGGCCAACTACTTCGAGAAGCCCAGTAACACCATGGAAGTTGCAGAGTGTTTCGCTCAGCACAACC
Case4  ----------------------------------------------------------------------------------------------------------------------------
Case5  ----------------------------------------------------------------------------------------------------------------------------
Case6  ----------------------------------------------------------------------------------------------------------------------------

Case2  CCAGTGTAGATCAGGTCGATGAGTCACTGCATACCCCACGGGTGACCGTGGCAGTGGCTGCGTTGGCGGCCTGCCTATGGGGAAGCCCATAGGACGCTCTAATACAGACATGGTGTGAAG
Case4  ----------------------------------------------------------------------------------------------------------------------------
Case5  ----------------------------------------------------------------------------------------------------------------------------
Case6  ----------------------------------------------------------------------------------------------------------------------------

Case2  AGTCTATTGAGCTAGTTGGTAGTCCTCCGGCCCCTGAATGCGGCTAATCCTAACTGTGGAGCATACGCCCTCAAGCCAGGGGGCAGTGTGTCGTAACGGGCAACTCTGCAGCGGAACCGA
Case4  ----------------------------CCCTGAATGCGGCTAATCCTAACTGTGGAGCACATGCCCACAAACCAGTGGGTGGTGTGTCGTAACGGGTAANTNNNNNGNNGAACCGA
Case5  ----------------------------------------------------------------------------------------------------------------------------
Case6  ----------------------------CCCTGANTGCGGCTAATCCTAACTGCGGAGCANATACCCACAAACCAGTGGGCAGTCTGTCGTAATGGGTAACTCTGCAGCGGAACCGA

Case2  CTAC------------------------------------------------------------------------------------------------------------------------
Case4  CTACTTTGGGTGTCCGTGTTTCTATTTATTCCTATACNGGC--------------------------------------------------------------------------------
Case5  ------------------------------------------------------------AANAGTAAAAGAAACACGGACACCCAAAGTAGTCGGTTCCGCTGCNNAGTTACCCATTACG
Case6  CTACTTTGGGTGTCCGTGTTTCT-TTTACTCTTTTATTGTCACCATAAGCAGCCAATAAAAGAGTAAAAGAAACACGGACACCCAAAGTAGTCGGTTCCGCTGCAGAGTTACCCATTACG

Case2  ----------------------------------------------
Case4  ----------------------------------------------
Case5  ACAGACTGCCCACTGGCTTGTGGGTNNCTGCTCCGCAGTTAGGATTA
Case6  ACAGACTGCCCAC-----------------------------------
```

Supplementary Table 5. Quality of RNA extracted for high-throughput RNA sequencing and generated sequences from pancreas of patients.

Case	A260/280	A260/230	RIN	Ribo-Zero input	Number of raw sequences (paired-end)	Number of quality-filtered sequences	% human rRNA*	% not mapping to human RNA/DNA*
1	2·10	2·20	8·5	1 µg	112507477	R1: 92733686 R2: 93298017	27·2	9·52
2	2·00	2·14	7·1	2 µg†	95513712	R1: 89023101 R2: 86592019	4·7	7·90
3	2·10	2·43	9·5	1 µg	143484083	R1: 119636360 R2: 119090740	22·0	9·79
4	2·10	2·02	9·2	1 µg	86350123	R1: 71492460 R2: 71703537	22·8	8·94
5	2·10	2·30	9·2	1 µg	124423978	R1: 105295210 R2: 105250837	3·5	11·92
6	2·15	2·30	8·4	2 µg†	103833945	R1: 93621816 R2: 90507443	46·7	6·38

*Using a random sample of 100k sequences from R1
†New Ribo-Zero Kit: Magnetic Gold
A260/280 and A260/230: wavelength ratios (from NanoDrop), RIN: RNA integrity value (from Bioanalyzer), R1 and R2: read 1 and 2 from paired-end sequences.

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