The enteroviruses detected in pancreatic samples belong to *Enterovirus B* species

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Purpose

Human enteroviruses (EVs) include >280 genotypes divided into seven species based on their genetic similarities. Species B EVs have been linked to type 1 diabetes (T1D) in several studies but the causality of this association and the underlying mechanisms have remained open. Species B EVs have tropism to the pancreas and to the insulin producing beta cells. They have damaged pancreatic islets in children who have died of EV infection (1, 2). In addition, beta cells express high levels of coxsackie and adenovirus receptor which is utilized by coxsackie B viruses (CBVs) (3). CBVs have also been diagnosed prior to the initiation of islet autoimmunity in cohort studies (4, 5). EVs have been detected in the pancreas of T1D patients but the serotype of these EVs has not been identified (except the isolation of CBV4 in two cases (1, 2)).

This is the first systematic study genotyping EVs that are present in the pancreas and pancreatic islets of T1D patients. Genotyping was done by sequencing the viral genome fragment that was amplified using a highly sensitive RT-PCR. In addition, the study compares pancreas-derived EV types to those detected in stool and blood samples of children who participated in the DIPP study.

Methods

Enterovirus RNA was screened from pancreas (nPOD, DiViD), stool (DIPP, DiViD) and serum (DIPP) samples using EV specific RT-PCR method targeting a part of the 5'UTR region. Further, positive samples were genotyped by sequencing a part of the viral VP1 protein coding region (6) and a part of the 5'UTR using traditional Sanger sequencing. The sequences were blasted against GenBank sequence database to genotype the detected viruses. The VP1 sequences were typed into genotype level and 5'UTR sequences in species level.

Genotyping of enteroviruses

The sensitivity of the RT-qPCR method that amplifies EV 5'UTR is more sensitive than that amplifying VP1 region. Therefore, the samples with low virus load (such as pancreatic samples) are difficult to sequence for the VP1 region even though they can be sequenced for 5'NCR. In stool samples which could be sequenced for both 5'UTR and VP1 regions a 66,7% concordance was seen in the genotyping results between 5'UTR and VP1 regions. In plasma samples the concordance rate was 71,4%. Similarly, the concordance in species level was 94,9% and 92,9%, respectively. This shows that 5'UTR sequences accurately classifies EVs into different species and it predicts also fairly well the genotype of these viruses

Results

Species A EVs are predominating EVs in serum and stool samples (VP1-sequence)

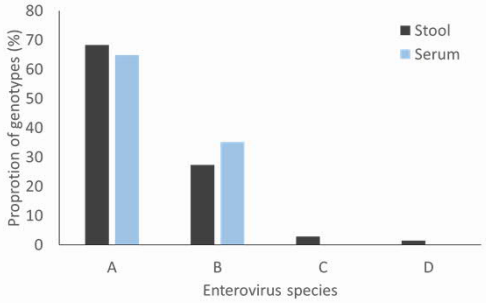

Figure 1. The proportions of EV species in stool and serum samples were typed for VP1 region. EV A types were the most common species both in stool and serum samples. EV B species were detected from both sample types, but EV C and D species were detected only from stool samples.

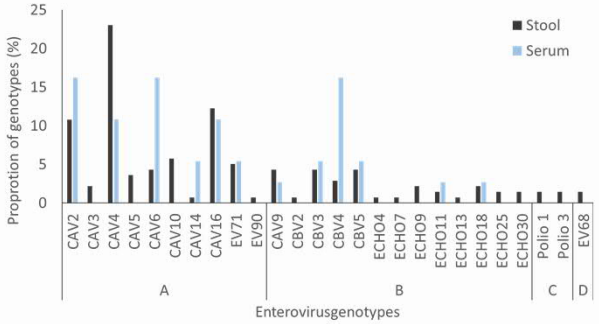

Figure 2. The proportions of EV genotypes in stool and serum samples were typed for VP1 region. From stool samples 26 genotypes were detected and the most common types were Coxsackievirus A4 (CAV4), CAV16, CAV2, CAV10 and EV71. In serum samples only 12 genotypes were detected and the most common types were CBV4, CAV2, CAV6, CAV16 and CAV4.

Species B EVs are predominating EVs in the pancreas (5'UTR sequence)

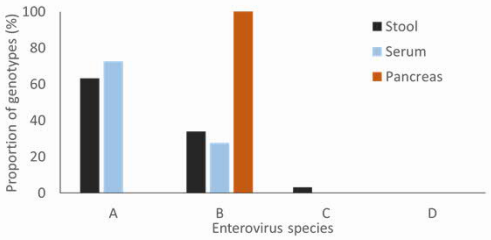

Figure 3. The proportions of EV species in stool, serum and pancreas samples were typed for 5'UTR region. EV A types were the most common species both in stool (63%) and serum (73%) samples. In contrast, all EVs in the pancreas samples were EV B species. The EV B species were detected also in stool (34%) and serum (28%) samples. In addition, 3% of EVs detected in stool samples were EV D species.

Summary of results

The genotype distribution differed markedly between the tested sample types. EV B species were more frequent in serum samples (35,1%) compared to the stool samples (27,3%) based on the VP1 coding region sequence. Similarly, analysis of 5'UTR region indicated that all EVs detected in the pancreas samples belonged to EV B species including CBV's, compared to 34% of EVs detected in the stool and 27% detected in serum samples. The most common species in stool (63%) and serum (72%) samples were EV A species.

Conclusions

Various EV genotypes were found in the stool samples, while more invasive infections were caused by a limited number of EV genotypes. Especially, in pancreas samples all the detected EVs belonged to the EV B species. The results support earlier findings of EV B infections being associated with the development of T1D. This could link to their property to cause invasive viremic infections that can spread to the blood and internal organs and their tropism to the pancreatic islets and beta cells. Vaccines against these viruses have prevented EV B viremia in mouse models and the ongoing development of CBV vaccine may tell if this is the case also in humans.

References

1. Yoon JW, Austin M, Onodera T, Notkins AL. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. 1979. N Engl J Med 300:1173-1179
2. Gladisch R, Hofmann W, Waldherr R. Myocarditis and insulitis following coxsackie virus infection. 1976. Z Kardiol 65:837-849
3. Ifie E, Russell MA, Dhayal S, Leete P, Sebastiani G et al. Unexpected subcellular distribution of a specific isoform of the Coxsackie and adenovirus receptor, CAR-SIV, in human pancreatic beta cells. Diabetologia. 2018. Nov;61(11):2344-2355. doi: 10.1007/s00125-018-4704-1. Epub 2018 Aug 3. PMID: 30074059; PMCID: PMC6182664.
4. Oikarinen S, Martiskainen M, Tauriainen S, Huhtala H, Ilonen J et al. Enterovirus RNA in blood is linked to the development of type 1 diabetes. Diabetes. 2011. Jan;60(1):276-9. doi: 10.2337/db10-0186. Epub 2010 Oct 13. PMID: 20943747; PMCID: PMC3012181.
5. Laitinen OH, Honkanen H, Pakkanen O, Oikarinen S, Hankaniemi MM et al. Coxsackievirus B1 is associated with induction of β-cell autoimmunity that portends type 1 diabetes. Diabetes. 2014 Feb;63(2):446-55. doi: 10.2337/db13-0619. Epub 2013 Aug 23. PMID: 23974921.
6. Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. 2006. *J Clin Microbiol*. 44(8):2698-2704. doi:10.1128/JCM.00542-06

This work was supported by grants from JDRF, European Commission (PEVNET project), Academy of Finland and Sigrid Juselius Foundation.

Conflict of interest statement: H.H. is the chairman of the board and a shareholder of Vactech Oy which develops vaccines against picornaviruses.