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Evaluation of RT-PCR and Immunohistochemistry as Tools for Detection of Enterovirus in the Human Pancreas and Islets of Langerhans

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Abstract

Background—Enteroviruses have been implicated in the etiology of type 1 diabetes, supported by immunoreactivity of enteroviral protein in islets, but presence of enteroviral genome has rarely been reported. Failure to detect enterovirus with RT-PCR has been attributed to the possible presence of PCR inhibitors and that only few cells are infected.

Objectives—The aim of this study was to evaluate strategies for detection of enterovirus in human islets.

Study design—A scenario was modeled with defined infected islets among a large number of uninfected pancreatic cells and the sensitivity of immunohistochemistry and PCR for detection of enterovirus was evaluated.

Results—Enterovirus was detected with PCR when only one single human islet, infected in vitro with a low dose of virus, was mixed with an uninfected pancreatic biopsy. Enterovirus could not be detected by immunohistochemistry under the same conditions, demonstrating the superior sensitivity of PCR also in pancreatic tissue with only a small fraction of infected cells. In addition, we demonstrate that pancreatic cell culture supernatant does not cause degradation of enterovirus

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Competing Interests

None declared

Ethical Approval

All work involving human tissue was conducted according to the principles expressed in the Declaration of Helsinki and in the European Council's Convention on Human Rights and Biomedicine. Consent for organ donation (for clinical transplantation and for use in research) was obtained verbally from the deceased's next of kin by the attending physician and documented in the medical records of the deceased in accordance with Swedish law and as approved by the Regional Ethics Committee. The study was approved by the Regional Ethics Committee in Uppsala, Sweden, according to the Act Concerning the Ethical Review of Research Involving Humans.

Contribution Statement

O.S. designed the study, researched data and wrote the manuscript. S.I. researched data and contributed to discussion and writing. O.K. designed the study and contributed to discussion and writing the manuscript. O.S. and O.K. are 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. All authors approved the final version to be published.

at 37°C, indicating that under normal culture conditions released virus is readily detectable. Utilizing PCR, the pancreases of two organ donors that died at onset of type 1 diabetes were found negative for enterovirus genome despite islet cells being positive using immunohistochemistry.

Conclusions—These data suggest that PCR should be the preferred screening method for enterovirus in the pancreas and suggest cautious interpretation of immunostaining for enterovirus that cannot be confirmed with PCR.

Keywords

Type 1 diabetes; etiology; pathogenesis; Coxsackievirus; VP1; infection; PCR; immunohistochemistry

Background

Type 1 diabetes has been associated with infection with Human Enterovirus (HEV), for several decades. This is a large group of viruses consisting of more than 70 serotypes and strains with diverse sequences and genetics and it is mainly those belonging to the group B Coxsackieviruses (CVB) that have been associated with type 1 diabetes [1-5]. The hypothesis that a direct HEV infection of the pancreatic islets contributes to the disease has been supported by demonstration of immunoreactivity of an antibody against a HEV capsid protein, VP1, in islets from diabetic subjects [6-10], but only rarely has HEV genome been demonstrated convincingly in the pancreas by in situ hybridization (ISH) [11] or virus isolation [3]. The use of reverse transcriptase (RT)-PCR, theoretically the most sensitive method for virus detection [12], has yielded negative results in pancreatic tissue [6, 13, 14]. In many studies, the only available material was formalin-fixed and paraffin-embedded (FFPE) pancreatic tissue in which the quality of RNA may be impaired to the extent that presence of viral RNA is not detectable with RT-PCR [15], but with recent focused initiatives to collect pancreatic tissues from diabetic subjects, such as the Network for Pancreatic Organ Donors with Diabetes (nPOD; www.jdrfnpod.org) [16], nPOD-Europe (www.jdrfnpod.org/europe.php), and EXODIAB in collaboration with the Nordic Network for Islet Transplantation (www.exodiab.se), the availability of pancreatic tissue suitable for RT-PCR analysis is increasing rapidly. A recent study of freshly frozen pancreatic tissue from the nPOD biobank found no HEV RNA despite using highly sensitive RT-PCR [14].

The failure to detect HEV RNA with RT-PCR has been attributed to the abundant presence of ribonuclease in pancreatic tissue, the possible presence of PCR inhibitors [17], and nonspecific amplification of sequences in the tissue that may outcompete virus-specific amplification [18]. It has also been suggested that only a minority of cells in the pancreas are infected in subjects with type 1 diabetes, reducing the likelihood of detection [19].

Objectives

The aim of this study was to develop a screening strategy for detection of enterovirus in human islets. Validation of the two techniques demonstrated that semi-nested (sn) RT-PCRs with primers in the 5'UTR and the VP1-3 regions were significantly more sensitive than IHC for detection of HEV in pancreatic tissue. Accordingly, we propose that PCR should be the preferred screening method for enterovirus in diabetic pancreata.

Study Design

Human Specimens

Human pancreases from two subjects with acute onset type 1 diabetes and two subjects without pancreatic disease were included in the study. They were procured from heart-beating multi-organ donors and transported to Uppsala in preservation solution minimizing post-mortal changes. Both subjects with type 1 diabetes died of brain edema and infarction in a series of complications associated with their type 1 diabetes debut, and their clinical data have been described previously [20]. Biopsies from the pancreas were immediately fixed in 4% PFA or immersed in liquid nitrogen. Pancreatic islets were isolated and kept as described previously [21].

In vitro infection

To compare the sensitivity of IHC and RT-snPCR in two different genomic regions, islets from organ donors without pancreatic disease were infected with a low dose (100TCID₅₀/200µL) of HEV (CVB4/VD2921; GenBank accession number AF328683) as described previously [22]. Twelve or 72 hours post infection (hpi) three islets were handpicked with a pipette under a light microscope, washed twice in 5 mL PBS, and added to; 1) 10 000 islets and 40 000 acinar cell clusters of similar size as human islets; 2) 30 mg frozen pancreatic biopsy homogenized in Buffer RLT Plus (Qiagen); or 3) an empty 1.5 mL tube. Total RNA was extracted with RNeasy Plus Mini kit (Qiagen) for RT-PCR analysis. Also, 100 islets were fixed in 4% PFA and processed for IHC.

Test of HEV stability and presence of RT-PCR inhibitors in pancreatic cell culture

Islets with low purity (~10%) and a total tissue volume of 150 µL were cultured in 200 mL culture media as described previously [21]. After 5 days the culture medium ("pancreatic cell culture supernatant") was filtered through a 0.45 µm filter to remove viable cells. Triplicates of a 10⁵ TCID₅₀/200 µl stock of HEV (CVB4/VD2921; GenBank accession number AF328683) were diluted 1:100 in fresh culture medium, or in the cell-free pancreatic cell culture supernatant, and viral RNA was extracted with Qiam UltraSens virus kit (Qiagen), directly, or after 24 h incubation at 37°C. The extracted RNA was stored at –80°C until semi-quantification with RT-qPCR.

Immunohistochemistry

6 µm sections from pancreatic biopsies and the in vitro-infected islets were processed for IHC [22]. A mouse monoclonal antibody clone 5-D8/1 VP1 (dilution 1:2000; DAKO, Glostrup, Denmark) was used for detection of HEV. Bound antibodies were visualized with DAKO EnVision™ (DAKO, Glostrup, Denmark).

Extraction of RNA from diabetic pancreata

RNA was extracted from isolated islets, cultured pancreatic cells (exocrine and endocrine), and pancreatic biopsies with RNeasy Plus Mini kit (Qiagen), and from pancreatic cell culture supernatants at several time points post isolation, with Qiam UltraSens virus kit

(Qiagen). The RNA extracted from tissue was analyzed by Bioanalyzer (Agilent 2100) and found to be of good quality (RIN values >9).

Enterovirus RT-snPCRs

Five µL of total RNA/sample were primed with HEV-specific primers (ECBV5 [23] in the 5'UTR region or AN32, AN33, AN34, and AN35 [18] in the VP1 region) and reverse transcribed to cDNA with SuperScript II RT (Invitrogen). Four µL of the cDNA was used in the first PCR (PCR#1) consisting of 10 µL 2X Taq MasterMix Plus (Qiagen), 2 µL CoralLoad (Qiagen), and 50 pmol each of primers 222 and 224 [18] (VP3-VP1 region) or primers ECBV5 [23] and EnteroF [24] (5'UTR), with 35 cycles of amplification (95°C for 30 s, 56°C for 30 s, 72°C for 20 s; 5'UTR) or with thermal cycling as described by Nix et al [18] (VP3-VP1). One µL of the first PCR product was added to a second PCR (PCR#2) for seminested amplification. PCR#2 was carried out as above but with 50 pmol each of primers EnteroF and EnteroR [24] (5'UTR) or 40 pmol each of primers AN89 and AN88 [18] (VP1) and 40 cycles of amplification (95°C for 20 s, 60°C for 20 s, 72°C for 15 s; 5'UTR) or as described in [18] (VP1). The sensitivity of sn RT-PCR for detection of enterovirus 5'UTR is <20 virus particles [12].

For quantitative PCR, 4 µL of cDNA, transcribed with primer ECBV5 [23], were amplified with primers EnteroF/EnteroR as described by Jonsson et al [24] with Power SYBR Green master mix (Applied Biosystems) on a StepOnePlus™ Real-Time PCR system (Applied Biosystems).

RT-PCR to detect virus in the pancreas

In addition to the highly sensitive RT-snPCRs for detection of HEV described above, the diabetic pancreata were examined for other picornavirus and other virus families with RT-PCR according to the methods described in the references given in Table 1.

Also, HEV were assayed with RT-snPCR with primers in the 3Dpol region (Forward: 5'-ATTCA YGAATCAATYAGATGGAC-3', and 5'-GCTCNYTGTGCTATTGGCTTGGCA-3', Reverse: 5'-ATAAGAATGCGGCCGCT_{27-3'}). The reverse primer was used for reverse transcription with SuperScript II RT (Invitrogen), and used together with the two forward primers separately in two rounds of seminested amplification (35 cycles each of 95°C for 30 s, 48°C for 45 s, 72°C for 30 s).

Virus isolation

Samples of isolated endocrine and exocrine tissue from the donors with type 1 diabetes were re-suspended in a phosphate buffer and inoculated onto GMK cells, RD cells, HeLa cells, as well as primary exocrine cells, cultures of isolated human islets, or to endocrine single cell culture from non-diabetic subjects. The inoculated cells were examined every day for the appearance of cytopathic effect. Samples of inoculated cells were subjected to RNA extraction and RT-snPCR with primers in the HEV 5'UTR as described above.

Results

Detection of HEV with RT-snPCR in a single infected islet

No positive cells could be detected with IHC with the antibody against HEV VP1 12 h after infection, however, a number of cells in the periphery of the islets were detected 72 h after infection (Fig. 1A and D). RT-snPCR on RNA from a single infected islet was strongly positive for HEV, both with primers in the 5'UTR (Fig. 1B and 1E) and in the VP3-VP1 region (Fig. 1C and 1F), irrespective of the time post infection. Already after the first round of RT-PCR (35 cycles), bands of the correct size could be visualized on agarose (Fig. 1B-C and 1E), except with primers in the VP3-VP1 region on RNA from an islet 12 hpi, which required snPCR to display positivity (Fig. 1F).

Co-extraction of RNA from one single infected islet mixed with a 30 mg pancreatic biopsy, or 10 000 non-infected islets and 40 000 acinar cell clusters of similar size as human islets, did not affect the sensitivity of the PCR (Fig. 1B-C and 1E-F), which was also confirmed by qPCR detecting HEV in the 5'UTR at Cq 30-33 and Cq 22-27 at 12 and 72 hpi respectively. Pancreatic tissue from the same donors was negative for HEV with PCR when no in vitro-infected islet was added.

Detection of HEV in cell culture supernatants with PCR is not inhibited by substances secreted from pancreatic cells

The presence of substances secreted to the cell culture supernatant by isolated islets and exocrine tissue did not inhibit the PCR reaction compared to fresh culture media (Fig. 2). Neither did the cell culture supernatant increase the degradation of HEV during incubation at 37°C for 24 h (Fig. 2).

Positive immunostaining for HEV capsid protein, but negative RT-snPCR for HEV RNA, in pancreas from two subjects with recent onset type 1 diabetes

Immunostaining for HEV VP1, in pancreatic biopsies from two organ donors that died at onset of type 1 diabetes, revealed intensely positive cells in multiple islets (Fig. 3). In biopsies from one of the donors, intense staining was seen in 3-87 cells/section and weaker staining in 5-30 cells/section. In biopsies from the other donor, intense staining was seen in 4-15 cells/section and a weaker staining in 0-41 cells/section.

No enteroviral RNA was detected in isolated islets, cultured pancreatic cells, pancreatic tissue biopsies, or pancreatic cell culture supernatants from the two donors with type 1 diabetes when using the highly sensitive RT-snPCRs described above. Also, RT-PCRs for detection of HEV, other Picornavirus, and other virus (Table 1) were all negative.

Virus isolation from islets, pancreatic tissue and islet culture medium from the two donors with type 1 diabetes was negative and no viral RNA could be detected in inoculated cells with RT-PCR.

Discussion

A screening strategy for detection of HEV in pancreatic biopsies is presented. The use of RT-snPCR has a theoretical possibility to detect 1 single copy of viral RNA [12, 25], whereas IHC requires a substantial number of virus particles to display distinct staining. In the present study, HEV was readily detected with RT-PCR when only one single islet, 12 h after infection *in vitro* with a low dose of HEV, was mixed with a pancreatic biopsy. The finding that the presence of a large number of uninfected pancreatic cells did not affect the number of PCR cycles needed to get a positive signal indicates that, with current protocols for RNA extraction and RT-PCR, neither PCR inhibitors in tissue nor unspecific amplification play any significant role. Even PCR with primers amplifying a fairly long (~950 bp) sequence of the HEV P3-P1 region was positive demonstrating that HEV RNA was not degraded under these premises. In contrast, IHC with the HEV VP1 antibody on these samples was negative, demonstrating the superior sensitivity of the PCR technology compared to IHC after enterovirus infection. In this study, the sensitivity was tested after acute *ex vivo* infection. Type 1 diabetic islets *in vivo* have been suggested to harbor a persistent, more low-grade, HEV infection [26], which is associated with restricted viral protein expression [27]. This likely reduces the sensitivity of IHC further, emphasizing the notion that RT-PCR should be the preferred screening method for HEV in the pancreas of subjects with type 1 diabetes.

Exocrine enzymes have been demonstrated to degrade HEV [28, 29], thereby limiting the possibility for detection. However, incubation with culture medium collected after 5 days of islet and exocrine *in vitro* culture did not increase HEV degradation at 37°C, demonstrating that under normal culture conditions viruses released to the culture medium are readily detectable.

The use of PCR, as well as IHC and ISH requires prior knowledge of the virus to be detected and HEVs are well known for their high variability. However, the 5'UTR of HEV is essential for replication and translation of the viral genome and contains nucleotide sequences with near absolute conservation among all enterovirus [30]. The use of these sequences for primer (RT-PCR) and probe (ISH) design allows detection of most known HEVs. Also, the use of degenerate inosine-containing primers and the consensus degenerate hybrid oligonucleotide primer (CODEHOP) approach developed by Nix et al. [18], and used in this present study, allows detection and genotyping in the more variable P1 region of all known HEV serotypes at high sensitivity. In IHC, conserved immunogenic regions in the P1 region are utilized for broad-spectrum detection of HEVs. The antibody used in this present study has been shown to react with most but not all HEVs [31, 32]. Because of the limitations with present methods for detection of virus in tissue, it is important that all results, positive as well as negative, are interpreted with caution and are confirmed with several different methods. Notably, RT-PCR is the only method that alone can prove the presence of virus in a sample since the specificity of the amplified product can be confirmed with sequencing, but instead it cannot be used to determine the location of the infected cell(s) in the tissue.

In the pancreata from the two subjects with recent onset type 1 diabetes, a large number of cells in multiple islets stained distinctly with an antibody against a HEV capsid protein, VP1. However, the negative findings using highly sensitive RT-snpCRs on well-preserved fresh frozen tissue from these subjects suggest that the mAb recognizes non-viral antigens, which has also been demonstrated for this mAb by previous reports [22, 33, 34]. Likewise, the presence of HEV was overestimated in hearts of subjects with myocarditis due to unspecific staining using immunohistochemistry [35],[36],[37]. Although it cannot be excluded that the antibody in our study detected a HEV not detectable with the primer pairs used, this does not seem likely given that several different primer pairs with broad reactivity were applied. Also, all the pancreatic samples were negative with PCR developed for detection of a number of other viruses. It should be noted however, that this study focused on the detection of HEV and that presence of the other viruses cannot be excluded since their positive controls were not included and these PCRs were not optimized for detection in pancreas. Novel methods, like next generation sequencing, with the capacity to detect viral genome without prior knowledge of the nucleotide sequence, may be necessary to find presence of tentative, hitherto unknown, viruses in the pancreases of subjects at onset of type 1 diabetes.

The herein described examination of fresh frozen pancreas from two organ donors that died at onset of type 1 diabetes did not reveal any support for the presence of viral genome. However, to unravel the potential role of HEV in the etiology of type 1 diabetes, biopsies from a larger number of subjects are needed. Also, depending on the mechanism by which HEV might induce diabetes, biopsies from pre-diabetic subjects may be required. In accordance with the broad reactivity and superior sensitivity of PCR compared to IHC, and since HEV protein expression is strictly dependent on presence of HEV genome, we propose that PCR should be the preferred screening method for assessing the presence of HEV in diabetic pancreata. However, precautions must be applied to avoid RNA degradation in clinical biopsies. If high amounts of virus are present, its localization within the specimen can be determined by ISH and/or IHC.

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List of abbreviations

FFPE	formalin-fixed paraffin-embedded
HEV	Human Enterovirus
ISH	in situ-hybridization

nPOD	Network for Pancreatic Organ Donors with Diabetes
sn	semi-nested
IHC	immunohistochemistry
UTR	untranslated region

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Highlights

- Potential PCR inhibitors in the pancreas do not affect sensitivity of the PCR assay.
- PCR is more sensitive and accurate than IHC for detection of enterovirus.
- Supernatants from pancreatic cell cultures do not show CVB4 degrading activity.
- Interpretation of IHC results that cannot be confirmed with PCR should be cautious.
- PCR should be the preferred method for screening and assessment of Enterovirus.

Figure 1. Detection of HEV in a single infected islet

A human islet, infected for 72 h (A-C) or 12 h (D-F) was mixed with non-infected cultured pancreatic cells, a pancreatic tissue biopsy, or used alone for RNA extraction. RT-snpPCR with primers in the 5' untranslated region (B, E) or in the VP3-VP1 region (C, F) was used for detection of HEV genome. The products from the first (PCR#1) and seminested (PCR#2) reactions were analyzed on a 1.5% agarose gel containing SYBR® Safe.

Immunohistochemical staining with an antibody against HEV VP1 was used for detection of HEV protein (A, D). A representative infected islet, infected for 72 h (A) or 12 h (D) is shown.

Figure 2. Degradation of HEV during incubation at 37°C

A 10^5 TCID₅₀/200 μ L stock of HEV was diluted 1:100 in fresh culture media, or cell-free pancreatic cell culture supernatant, and viral RNA was extracted directly, or after 24 h incubation at 37°C. The amount of viral RNA was semi-quantified with qPCR and the Cq values of three independent experiments are displayed on the vertical axis. Cq; quantification cycle.

Figure 3. Immunoreactivity of an antibody against a HEV capsid protein, VP1, in islets at onset of type 1 diabetes

Immunohistochemical staining with an antibody against HEV VP1 (5-D8/1 DAKO) of a pancreatic section from an organ donor that died at acute onset type 1 diabetes. Original magnification X20.

Table 1

PCRs used for detection of virus in the pancreas from the two subjects with recent onset type 1 diabetes.

Virus	Amplified region	Reference
Human Enterovirus	5'UTR	[23, 24]
Human Enterovirus	VP3-VP1	[18]
Human Enterovirus	5'UTR	[23]
Human Enterovirus	3Dpol	
Ljungan and Human Parecho	5'UTR	[38]
Ljungan	5'UTR	[39]
Cosavirus	5'UTR	[40]
Cardiovirus	5'UTR	[41]
Echovirus 22 and 23	5'UTR	[42]
Hepatitis A virus	VP1	[43]
Rotavirus A	VP7	[43]
Rotavirus B/C	NSP2/VP6	[44]
Human Herpesvirus 6		[45]
Herpes Simplex virus 1 and 2	U38	[46]
Varicella-zoster virus	ORF29	[46]
Human Cytomegalovirus	U38	[46]
Epstein-Barr virus	U38	[46]