Establishing Evidence for Enterovirus Infection in Chronic Disease

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ABSTRACT: Viruses have long been considered among potential environmental triggers of type 1 diabetes mellitus. Epidemiologic and seroprevalence studies have associated enterovirus infection with development of prediabetic autoimmunity and with the onset of clinical diabetes. Enterovirus infection has also been temporally correlated with disease onset by virus isolation or by detection of viral genome by reverse transcription–polymerase chain reaction (RT-PCR). For the large-scale prospective studies that are required to firmly establish a causal relationship between enterovirus infection and development of prediabetic autoimmunity or progression from autoimmunity to clinical diabetes, sensitive RT-PCR methods must be used to detect virus prior to the onset of diabetic symptoms. We have developed an RT-seminested PCR protocol to detect enteroviruses in clinical specimens. This method is approximately 10,000-fold more sensitive than conventional, single-amplification PCR. Further, we have developed molecular methods to rapidly and reliably identify enterovirus serotype, bypassing the cumbersome and often problematic neutralization test. The molecular serotyping approach will be valuable in examining the relationships between particular virus serotypes or genotypes and specific diseases.

KEYWORDS: enterovirus; type 1 diabetes mellitus; RT-PCR; seminested PCR; molecular typing

While host genetic determinants have a major influence on an individual's risk of developing type 1 diabetes mellitus (T1DM), environmental factors, such as foods and infectious agents, are thought to play a role in the genesis of prediabetic autoimmunity or in the progression from persistent beta cell autoimmunity to clinical diabetes.[1,2] Immunity to one or more beta cell autoantigens, such as insulin, GAD65, or IA-2, may lead to destruction of beta cells and a loss of the capacity to produce insulin, ultimately resulting in clinical insulin-dependent diabetes mellitus. Postulated mechanisms by which infectious agents may trigger T1DM include (i) direct cytolytic infection of beta cells, resulting in destruction of beta cells and loss of capacity to synthesize insulin; (ii) a virus-induced immune response against infected beta cells, such as T cell–induced killing of virus-infected cells; (iii) nonspecific "innocent

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bystander" killing of beta cells through activation of nonspecific immune mediators; and (iv) induction of an autoimmune response to islet antigens by cross-reactivity with viral antigens (molecular mimicry) or disruption or normal immune tolerance mechanisms.

Several viruses have been proposed as infectious triggers of diabetes, but the enteroviruses (family *Picornaviridae*, genus *Enterovirus*) are the subject of the most intense scrutiny at present.[3,4] Numerous studies have provided evidence for an association between enterovirus infection and prediabetic autoimmunity or clinical diabetes. Diabetes incidence has been epidemiologically linked to the incidence of enteroviral meningitis or enterovirus outbreaks.[5] Serologic studies have shown that there is a correlation between enterovirus seroprevalence in patients with prediabetic autoimmunity or diabetes compared to unaffected control individuals.[6,7] Direct enterovirus detection in pancreas, blood, serum, or stool has suggested a temporal correlation between enterovirus infection and onset of diabetes.[8–10]

Enteroviruses are among the most common of human viruses, infecting an estimated 50 million people annually in the United States and possibly a billion or more annually worldwide.[11,12] Most infections are inapparent, but enteroviruses may cause a wide spectrum of acute disease, including mild upper respiratory illness (common cold), febrile rash (hand-foot-and-mouth disease and herpangina), aseptic meningitis, pleurodynia, encephalitis, acute flaccid paralysis (paralytic poliomyelitis), and neonatal sepsis-like disease. Enterovirus infections result in 30,000 to 50,000 hospitalizations per year in the United States, with aseptic meningitis cases accounting for the vast majority of the hospitalizations.[12] In addition to these acute illnesses, enteroviruses have also been associated with severe chronic diseases such as myocarditis,[13,14] T1DM,[3,15] and neuromuscular diseases.[16] Enteroviruses are transmitted primarily by the fecal-oral route, but respiratory transmission to close contacts may also be important. The incubation period between infection and onset of symptoms is usually 4–7 days. The intestinal mucosa or upper respiratory tract is the site of primary infection, with secondary spread to the central nervous system and other tissues. Viremia is usually short-lived, often waning before the onset of symptoms, except in very young children. Virus is excreted in the stool for up to 8 weeks (average 2–4 weeks), but maximal virus shedding occurs before the onset of symptoms. The maximum virus titer in stool is ~10^4 infectious virus particles per gram.

Of the 89 recognized enterovirus serotypes, 64 are known to infect humans.[12] In addition to the human enteroviruses, human pathogenic viruses are found in 4 other picornavirus genera: *Rhinovirus* (human rhinoviruses), *Hepatovirus* (human hepatitis A virus), *Parechovirus* (human parechoviruses 1 and 2, formerly echoviruses 22 and 23, respectively), and *Kobuvirus* (aichivirus, an agent of gastroenteritis). Most of the human enterovirus serotypes were discovered and described between 1947 and 1963 as a result of the application of cell culture and suckling mouse inoculation to the investigation of cases of infantile paralysis (paralytic poliomyelitis) and other central nervous system diseases.[17,18] The human enteroviruses were originally classified on the basis of human disease (polioviruses), replication and pathogenesis in newborn mice (Coxsackie A and B viruses), and growth in cell culture without causing disease in mice (echoviruses), but they have recently been reclassified, based largely on molecular properties, into four species, A through D.[19] Sequences in various portions of the enterovirus coding region correlate with species, but only capsid sequence correlates with serotype.

The neutralization test, long the "gold standard" for enterovirus typing, is generally reliable, but it is labor-intensive and time-consuming, and may fail to identify an isolate because of aggregation of virus particles or antigenic drift (the widely used standardized typing antisera were raised against prototype strains that were isolated 40 to 50 years ago[20]). Antisera to all serotypes are not generally available and isolates that are not of a known human enterovirus serotype (new serotypes or serotypes that normally infect animals other than humans) would obviously also present difficulties in identification by antigenic means as the neutralization method requires the use of serotype-specific reagents. In addition, neutralization requires virus isolation, which may require the use of multiple cell lines and adds to the time required to make an identification.

The application of PCR has improved the speed and accuracy of general enterovirus detection[21,22] and has found wide acceptance in the clinical diagnostic laboratory. Since the enterovirus serotype is rarely relevant to clinical case management, many clinical virology laboratories are bypassing virus isolation entirely in favor of PCR detection of viral nucleic acid directly in clinical specimens such as cerebrospinal fluid, nasopharyngeal swabs, or tissue specimens.[21] This approach uses genus-specific primers targeted to the 5′-nontranslated region (FIG. 1), often coupled to probe-hybridization and detection of product in a microplate format.[21] Specimens of choice for the direct detection of enteroviruses by RT-PCR are stool or rectal swab (stool is preferred because it contains a larger amount of fecal material and, hence, virus), oro- or nasopharyngeal specimens (throat swab, nasopharyngeal swab or aspirate, saliva), cerebrospinal fluid (if there is concomitant CNS disease), fresh-frozen or formalin-fixed tissue, and serum/plasma. Serum and plasma are generally

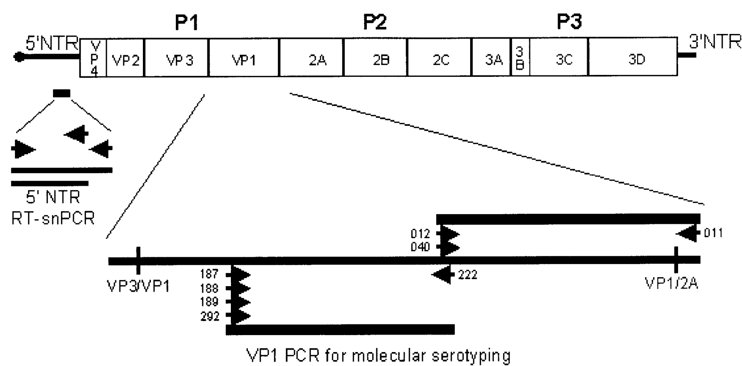


FIGURE 1. Schematic representation of the enterovirus genome, indicating regions that have been targeted for development of PCR diagnostics. The genome is a positive-stranded, polyadenylated RNA of ~7400 nucleotides, with a viral protein (3B/VPg) covalently linked to the 5′-end. The genome is divided into five functional regions: the 5′-nontranslated region (NTR) (control of viral translation initiation and initiation of positive-strand RNA synthesis); P1 (encodes the structural proteins that comprise the virus capsid); P2 and P3 (encode the nonstructural proteins involved in RNA replication, proteolytic processing of polyprotein, and host cell shutdown); and 3′-NTR (involved in initiation of negative-strand RNA synthesis).

only useful for RT-PCR in infants because viremia may still be present after onset of symptoms. If virus is detected only in a nonsterile site, such as stool or nasopharynx, a large number of patients are needed to establish the association between infection and disease.

Despite the advantages of enterovirus detection by RT-PCR, challenges remain. In the case of chronic diseases, the virus may act indirectly (e.g., through immune-mediated pathology). The virus may be cleared well before disease onset or virus may be present in the patient, but not in the diseased tissue. Even in acute illnesses, the titer is relatively low in all specimens. As a result, a "conventional" single-step RT-PCR amplification may not be sensitive enough for direct detection from the original clinical specimen. Designing a prospective study and collecting multiple specimens, at multiple time points throughout the duration of the study, may overcome some of these problems; however, the only way to solve the sensitivity problem is by increasing the sensitivity of the detection method. To address this issue, we have developed an enterovirus-specific seminested RT-PCR assay (5′-NTR RT-snPCR) that targets the conserved regions of the 5′-NTR (FIG. 1). FIGURE 2 shows the sensitivity of our standard, conventional RT-PCR[23] compared with that of the 5′-NTR RT-snPCR. Tenfold serial dilutions of a virus isolate (10^{-1} to 10^{-10}) were prepared with uninfected cell extract as diluent. RNA was extracted using the QIAamp viral RNA mini-kit (Qiagen, Inc., Valencia, CA) and reverse-transcribed using the antisense primer. PCR was performed using a single round of amplification (conventional PCR) or two rounds of amplification (seminested PCR). The second round of the seminested amplification used the same primers as the conventional

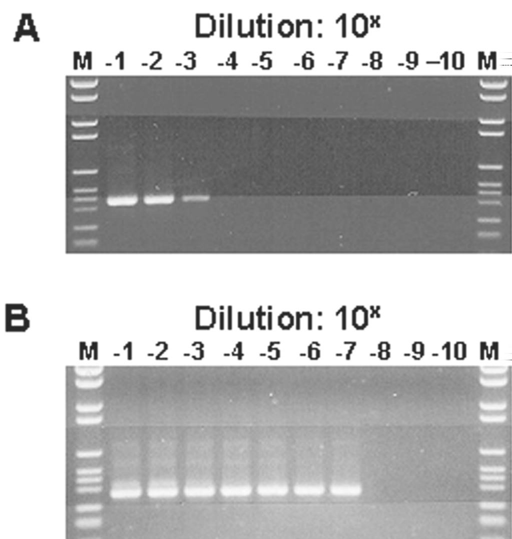


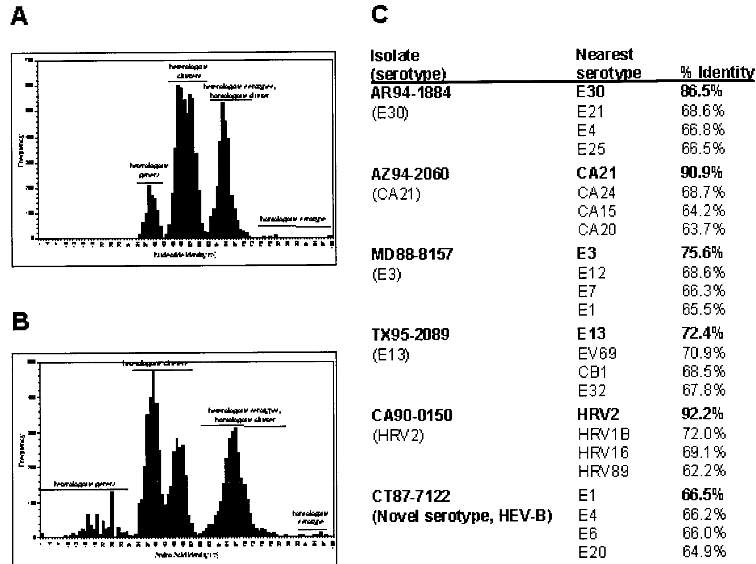
FIGURE 2. Sensitivity of pan-enterovirus RT-PCR methods; M, molecular weight marker. Virus dilutions are shown at the top of each panel. **(A)** Titration of conventional two-primer RT-PCR. **(B)** Titration of RT-seminested (three-primer) PCR.

PCR. Amplification products were visualized by polyacrylamide gel electrophoresis and staining with ethidium bromide. The RT-snPCR method (FIG. 2B) was ~10,000-fold more sensitive than the conventional RT-PCR (FIG. 2A). The 10^{-7} dilution corresponds to less than 20 infectious virus particles.

Enterovirus infection elicits a serotype-specific immune response directed against epitopes on the surface of the viral capsid. Mucosal immunity is most important. Antibody alone fully protects from disease, probably by limiting virus spread from the gut, but antibody does not necessarily protect from infection. The virus-specific T cell response, directed against epitopes on both the structural and non-structural proteins, is probably involved in virus clearance, but it is not needed for protection. Antigenic sites are located in each of the three enterovirus structural proteins, VP1, VP2, and VP3,[24,25] but the epitopes responsible for serotype specificity have not been identified. Since the picornavirus VP1 protein contains a number of immunodominant neutralization domains, we hypothesized that VP1 sequence should correspond with neutralization properties (serotype).[26] Due to the high frequency of recombination among picornaviruses,[27–29] sequence information from noncapsid regions is of little value in characterizing new serotypes within known genera.

VP1 sequence relationships within a serotype, within a species, between species, and between human enteroviruses and other picornaviruses were analyzed by comparison of the nucleotide and deduced amino acid sequences of all possible human enterovirus VP1 sequence pairs.[26] The relationships were visualized by plotting the frequency of pairwise identity scores versus percent identity, rounded down to the nearest integer value, as a histogram (FIG. 3). For both the nucleotide (FIG. 3A) and amino acid (FIG. 3B) pairwise identity distributions, the scores fell into four categories. The highest scores (nucleotide identity of ≥75%; amino acid identity of ≥85%) depict relationships among viruses of the same serotype. Nucleotide identity scores for pairwise comparisons within a species ranged from 48.9% to 73.2% and defined a peak that was clearly delineated from the homologous pairs and from the peak of scores comparing viruses of different species (FIG. 3A). Scores among viruses in *Human Enterovirus A* (HEV-A) ranged from 58.5% to 73.2%, while those among HEV-C viruses ranged from 55.9% to 70.6%. Viruses in HEV-B appeared to be somewhat more heterogeneous, with scores ranging from 48.9% to 71.8%. Scores in the heterologous comparison peak ranged from 42.1% to 64.5% nucleotide identity and depict relationships between serotypes of different species. The final peak, containing the lowest scores, represented comparisons of viruses of different genera within the family Picornaviridae. In the amino acid identity distribution (FIG. 3B), the heterologous species peak appeared to be composed of two overlapping peaks. The peak with higher scores represented comparisons of viruses from phylogenetically related species (e.g., HEV-B and HEV-C), whereas the peak with lower scores represented comparisons among viruses of more distantly related species (e.g., HEV-A and HEV-B).

Practical criteria must be established before molecular sequence information can be applied routinely to picornavirus identification. A partial or complete VP1 nucleotide sequence identity of at least 75% (minimum 85% amino acid sequence identity) between a clinical enterovirus isolate and serotype prototype strain may be used to establish the serotype of the isolate.[26,30,31] These criteria also appear to apply to comparisons among isolates of foot-and-mouth-disease virus (family Picornaviridae, genus *Aphthovirus*),[32] but a study directly comparable to the enterovirus studies has



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Isolate (serotype)	Nearest serotype	% Identity
AR94-1884	**E30**	**86.5%**
(E30)	E21	68.6%
	E4	66.8%
	E25	66.5%
AZ94-2060	**CA21**	**90.9%**
(CA21)	CA24	68.7%
	CA15	64.2%
	CA20	63.7%
MD88-8157	**E3**	**75.6%**
(E3)	E12	68.6%
	E7	66.3%
	E1	65.5%
TX95-2089	**E13**	**72.4%**
(E13)	EV69	70.9%
	CB1	68.5%
	E32	67.8%
CA90-0150	**HRV2**	**92.2%**
(HRV2)	HRV1B	72.0%
	HRV16	69.1%
	HRV89	62.2%
CT87-7122	E1	**66.5%**
(Novel serotype, HEV-B)	E4	66.2%
	E6	66.0%
	E20	64.9%

FIGURE 3. Frequency distribution of pairwise identity scores for comparison of VP1 nucleotide and deduced amino acid sequences. **(A)** Nucleotide sequence distribution. **(B)** Amino acid sequence distribution. **(C)** Example of the application of partial VP1 sequence comparisons to the identification of clinical isolates.

not yet been performed. A best-match nucleotide sequence identity of between 70% and 75% or a second-highest score of greater than 70% may provide a tentative identification, pending confirmation by other means, such as neutralization with monospecific antisera[31] or more extensive sequencing. A best-match nucleotide sequence identity below 70% (less than 85% amino acid sequence identity) may indicate that the isolate represents an unknown serotype.[31,33] Sequencing of the complete capsid coding region may be useful in confirming this result, but complete capsid sequences are available for less than half of the known enterovirus serotypes, limiting the utility of complete capsid sequence comparisons until more sequence becomes available. More extensive characterization, possibly including complete genome sequences, may be required for viruses that appear to represent previously unknown genera.[34–37]

Recognizing the technical difficulties and limitations inherent in the classic approach to enterovirus identification, we developed RT-PCR and sequencing primers that target the VP1 capsid gene and may be used to determine enterovirus serotype by sequencing of the amplicon and comparison to a database of the VP1 sequences of all enterovirus serotypes.[26,30,31] Our first set of primers amplified the prototype strains of 44 of the 64 enterovirus serotypes, as well as clinical isolates of several additional serotypes. These primers (012-040-011) amplify a product of ~450 bp corresponding to the 3′-half of VP1 (FIG. 1),[30] but they failed to amplify some of the prototype strains, primarily because of variability in the annealing site of primer 011 in 2A. The failure of primers 012-040-011 to amplify all enterovirus serotypes

limited their usefulness in routine diagnostic testing.[30,31] Analysis of the complete VP1 amino acid sequences of all 64 enterovirus serotypes revealed 2 amino acid motifs that were highly conserved among all serotypes, corresponding to VP1 amino acids (NQT)A(AV)ETG and M(FIY)VPPG, respectively. A primer set targeting the sites encoding these motifs was developed for identification of isolates that were not amplified by 012-040-011.[31] Primers 187, 188, and 189 anneal to analogous sites encoding the (NQT)A(AV)ETG motif in viruses of HEV-B, HEV-C and -D, and HEV-A, respectively (FIG. 1). Primer 292 represents a consensus of 187, 188, and 189 (FIG. 1). Primer 222 anneals to the site encoding the M(FIY)VPPG motif. Primers 187-188-189-222 (and 292-222) amplify all enterovirus serotypes, producing a PCR product of about 350 nucleotides and allowing the simple and rapid identification of any enterovirus isolate. Some human rhinoviruses may also be amplified. An example of the application of this system is shown in FIGURE 3C. The highest scores for 4 of the isolates (AR94-1884, AZ94-2060, MD88-8157, and CA90-0150) were greater than 75%, ranging from 75.6% to 92.2%. These high scores were clearly resolved from the second-highest scores, which were 68.2% to 72.0%. By contrast, the high score for TX95-2089 was only 72.4% (to echovirus 13) and the second-highest score was 71.9% (to enterovirus 69). Similar scores were obtained with complete VP1 sequence. However, the isolate was fully neutralized by anti-echovirus 13 antisera, but not by antisera to any of the 3 next-highest-scoring serotypes.[30] For CT87-7122, all of the scores were less than 67% and it was not neutralized by any of the antisera,[31] strongly suggesting that it represents a new enterovirus serotype. Since 1998, over 1200 isolates of 57 different serotypes have been identified in our laboratory using the molecular serotyping approach. Only CA1, CA7, CA11, CA19, CA22, E31, and EV69 were never encountered.

These molecular detection and typing methods, when coupled with well-designed prospective studies, will be useful in addressing the potential causal relationship between enterovirus infection and development of prediabetic autoimmunity or progression from persistent autoimmunity to clinical diabetes.

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