Chapter 13
Laboratory Diagnosis of Enterovirus Infection: Optimal Methods for Studies of Diabetes

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Abstract Laboratory diagnosis of enterovirus infections is more complex than that of many other virus infections. Careful design of study protocols and sample collection procedures is crucial for studies evaluating the role of enteroviruses in type 1 diabetes. Possible viral persistence creates an additional challenge, since the virus may be present in low quantities and in the form of double-stranded RNA. Both direct virus detection and serology have their own advantages and disadvantages, depending on the individual research questions, technologies, and sample types used in the studies. In many cases, their combined use would give the best view on the relationship between enteroviruses and type 1 diabetes. Standardization of enterovirus assays by international collaboration would help identify the optimal diagnostic approaches for type 1 diabetes studies.

Introduction

Enteroviruses have been linked to type 1 diabetes in various stages of beta-cell damaging process, including the time when the autoimmune process begins (the detection of first autoantibodies), during the progression of this process in children with autoantibodies, and at the onset of clinical diabetes (Stene et al. 2010; Oikarinen et al. 2011; Yeung et al. 2011). In addition, some studies have indicated that maternal infections during pregnancy may increase the risk of type 1 diabetes in the child (Hyöty et al. 1995; Elfving et al. 2008). These studies have been based on the "direct" detection of viral RNA or virus proteins in different sample types from prediabetic and type 1 diabetes patients or on the "indirect methods" detecting the markers of human immune response induced by virus infection (Yeung et al. 2011).

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Reliable detection of enteroviruses in clinical samples is more challenging compared to the detection of many other viruses, mainly because of the high diversity of enteroviruses. They include more than 100 different serotypes with considerable genetic variation. The classification of enteroviruses has been revolutionized by the implementation of molecular methods which can identify genetic relationships by sequencing the viral genome (Oberste et al. 1999a, b). These methods have identified over 30 new enterovirus types and their number is continuously increasing.

Detection of Enteroviruses

Optimal Samples

The majority of the studies addressing the role of enteroviruses in type 1 diabetes have been carried out at the time of diagnosis of the disease. However, it would be essential to study factors that trigger the type 1 diabetes process and which can occur several years before the clinical disease is manifested. Critical infections may be experienced in early infancy or even in utero during pregnancy, and therefore prospective sample series covering the time from birth to the diagnosis of type 1 diabetes would be optimal. In addition, such longitudinal sample series should be taken at relatively short intervals and cover different kinds of sample types where enteroviruses can be detected. Blood and stool samples are usually used when diagnosing enterovirus infections by direct virus detection or antibody assays. In the case of persisting and slowly replicating infection, direct detection of the virus would be particularly challenging, and additional sample types such as tissue samples may be needed. However, it is difficult to obtain biopsies from the pancreas for this type of studies, and such samples are available only in exceptional cases (Imagawa et al. 2001). Prospective studies are very expensive and time consuming, and need a well-organized infrastructure for clinical follow-up. Since the predictive value of the best diabetes risk markers is still far below 100%, a large number of originally non-diabetic subjects have to be followed-up and only few will develop diabetes. So far, extensive prospective studies have been carried out in a few countries including Finland (DIPP and DiMe studies), Germany (BabyDiab study), the USA (DAISY study), and Norway (MIDIA study). Recently, a multicenter TEDDY study has been started in the USA, Finland, Sweden, and Germany. Most of these studies have focused on children who have increased genetic risk for type 1 diabetes and these children have been followed-up from birth.

The selection of the appropriate sample material is crucial for optimal detection of enteroviruses and their possible association with type 1 diabetes. The primary replication of enteroviruses occurs in the intestinal and respiratory mucosa. Infection may be limited to the mucosal surfaces, but in many cases the virus spreads to the blood causing primary viremia. Subsequently, virus may spread to secondary replication sites such as the pancreas and later a secondary episode of viremia may occur. Viremia is short and usually lasts from a few days to no more than 2 weeks. Thus, such a short period reduces the possibility of detecting the virus in the blood, particularly because

enterovirus infections are usually asymptomatic and sampling can only be guided occasionally by typical symptoms (Racaniello 2001). This creates a big challenge particularly in prospective studies where blood samples are taken regularly according to a predetermined schedule with relatively long intervals. Viremia can be detected by virus isolation or by PCR (viral RNA in blood). Virus isolation from blood has not been widely used in diabetes studies, but viral RNA has been detected using PCR in whole blood samples as well as in serum or plasma samples taken from type 1 diabetic patients more frequently than in control subjects. In addition, viral RNA has been detected in serum and plasma taken from prediabetic individuals in prospective studies (Tauriainen et al. 2010; Yeung et al. 2011).

In addition to acute infection, viremia may also occur in persistent infection. In such a case, infectious virus is not necessarily present but the virus may be detectable in white blood cells at the RNA or double-stranded RNA level. Some studies have suggested that enteroviruses can be detected in antigen presenting cells in patients with type 1 diabetes (Schulte et al. 2010). These observations suggest that samples enriched for antigen presenting cells might be one of the most optimal targets for virus detection in the peripheral blood of patients with type 1 diabetes.

Enteroviruses are common in young children and are often detected in stool samples collected at random from healthy children. Thus, there is a risk that background infections may mask the possible risk effect of diabetogenic enterovirus types, especially in countries where enterovirus infections are common. In fact, even though enteroviruses have been detected more frequently in the blood of patients with type 1 diabetes than in control subjects, no such difference has been found in stool samples. In a recent study enterovirus was detected equally frequently in stool samples collected from children who developed islet autoantibodies as in control children (Tapia et al. 2011). It is also possible that the frequency of enterovirus infections is about the same in healthy and type 1 diabetes cases, but the susceptibility to the diabetogenic effect of the virus differs between the groups. In such scenario, the risk effect of enteroviruses would be detectable in the subgroup of children who carry this susceptibility (e.g., certain risk genes such as IFIH1).

Detection of enteroviruses in the primary and secondary replication sites, such as biopsies from the intestine and the pancreas, would provide important additional information about type 1 diabetes process. Detection of the virus in the pancreatic islets would be particularly important since it would provide a biological explanation for the islet inflammatory process which is the hallmark of type 1 diabetes. However, due to the anatomic location of the pancreas, it has been difficult to obtain such samples. Currently, large-scale international studies are in progress to collect such samples from prediabetic and diabetic subjects (nPOD study organized in the USA and euroPOD in Europe).

Detection of Viral RNA by RT-PCR

RT-PCR offers certain important advantages for studies evaluating the viral etiology of type 1 diabetes. It is generally more sensitive than other methods used for the detection of viruses directly in clinical specimen. In addition, it makes it possible to

study the molecular structure of the viral genome by sequencing PCR amplicons. PCR methods which specifically amplify enterovirus RNA genome have been used widely for detection of the virus in the blood, stool, and tissue samples of patients with type 1 diabetes. These studies have found the virus more frequently in the diabetic patients than in controls (see the recent meta-analysis by Yeung et al. 2011). The sensitivity of virus-specific PCR assays is usually better than that of the new next-generation sequencing methods which are becoming more and more popular, making it possible to detect a wide range of different microbes in a single test. High sensitivity is an important goal because the amount of the virus can be very low due to the nature of the infection such as persistent infection or available sample types, e.g., blood and tissue samples.

Optimization of PCR-based methods is critical for reliable detection of enteroviruses. The assay should be sensitive and specific yet relatively insensitive to PCR inhibitors (Oikarinen et al. 2009). International quality control programs such as Quality Control for Molecular Diagnostics (QCMD) have shown that the sensitivity of enterovirus PCR varies widely between different laboratories, and samples with low virus load are not detected by all methods. These types of quality control panels have indicated that virus laboratories can also have problems with contamination, which may lead to false-positive findings. To achieve high quality in PCR-based diagnosis, the different steps of the PCR assay (RNA extraction, RT and PCR enzymes and reaction conditions, and the sequences of primers) must be optimized and adjusted with the genetic variation of enteroviruses, type of infection to be diagnosed (acute vs. persistent), and sample material available (e.g., amount of PCR inhibitors in the sample). In addition, the technical performance of PCR should be monitored by positive, negative, and internal control samples included in each PCR run. The life cycle of enteroviruses may have relevance in diabetes research, since there is some evidence that the virus may persist in double-stranded RNA form in the pancreas or other tissues in patients with type 1 diabetes and/or the RNA genome may also be in the negative strand form (Klingel et al. 1992; Richardson et al. 2011). In such cases pre-heating of the RNA sample to denaturate double-stranded RNA and use of both sense and antisense primers in the RT-PCR may be important. In addition, deletions may be present in the 5′UTR region of the genome of persisting virus variants (Chapman et al. 2008). Such deletions could have critical consequences, if the primer annealing site is located in that region.

Molecular Typing of Enteroviruses

Enteroviruses cluster into two groups based on their 5′UTR region. On the other hand, the nonstructural gene regions are species-specific, i.e., enteroviruses are clustered into four genogroups A–D. If genotyping is used to identify the serotype of the virus (a correlate of traditional serological typing) the capsid protein coding regions VP1–VP4 should be sequenced. In this region, the intragenotypic divergence in the nucleotide level is up to 25% and in amino acid level up to 12%

(Oberste et al. 1999a, b). This is, of course, an advantage for typing itself, but a disadvantage for primer design, because the use of wobble nucleotides and the long length of resulting PCR amplicons decrease the sensitivity of PCR. The most reliable region for the genotyping of enteroviruses is the VP1 region which contains the major antigenic sites (Oberste et al. 1999a, b; Nix et al. 2006). All known enteroviruses have been sequenced using this region and the sequences can be found in the GenBank. Therefore, VP1 sequencing is becoming more and more important for genotyping of enteroviruses. Genotyping is often performed using blast search, but deeper phylogenetic analysis provides more reliable results. For successful phylogenetic analysis expertise and understanding of different methods as well as the influence of different assay parameters are needed. For example, (Kroneman et al. 2011) have published Web-based genotyping tools using optimized algorithms and parameters for phylogenetic analyses.

Virus Isolation

Enteroviruses can be isolated from several types of samples, including tissue and blood samples as well as respiratory secretions and stools. The concentration of the virus is the highest in stool and in respiratory secretions (e.g., throat swabs). Stool samples are widely used as primary samples for enterovirus isolation. In some cases, enteroviruses can be detected in stool samples for prolonged time periods after infection, ranging up to several weeks or even months. However, this is not true for all infections as certain enteroviruses are preferentially excreted via respiratory route. The main advantage of virus isolation is the possibility of using the isolated virus strains in further experimental studies in different model systems and to characterize them molecularly in detail (complete sequence). For example, virus strains isolated at the start of the beta cell damaging process could be studied in human pancreatic islet-cell cultures to see possible specific interactions with these cells. The main disadvantage of virus isolation is its relatively low sensitivity compared to PCR. In addition, many enterovirus serotypes do not grow well in cell lines, a fact that may cause false negative results. Virus isolation is also labor-intensive requiring sterile cell culture work. Therefore, PCR has largely replaced virus isolation in diagnostic laboratories. In diabetes research, PCR can be done first, and virus isolation can be attempted from PCR positive samples. However, it should be noted that in samples which contain large concentrations of PCR inhibitors, the sensitivity of virus isolation can actually be better than that of PCR. Such inhibitors are frequent, for instance, in stool samples (Oikarinen et al. 2009).

Tissue Tests

Immunohistochemistry (IHC) and in situ hybridization (ISH) are the methods most commonly used for detection of enteroviruses in tissue samples (Fig. 13.1). In addition, RT-PCR can be used, even though its sensitivity may not be optimal in formalin-fixed

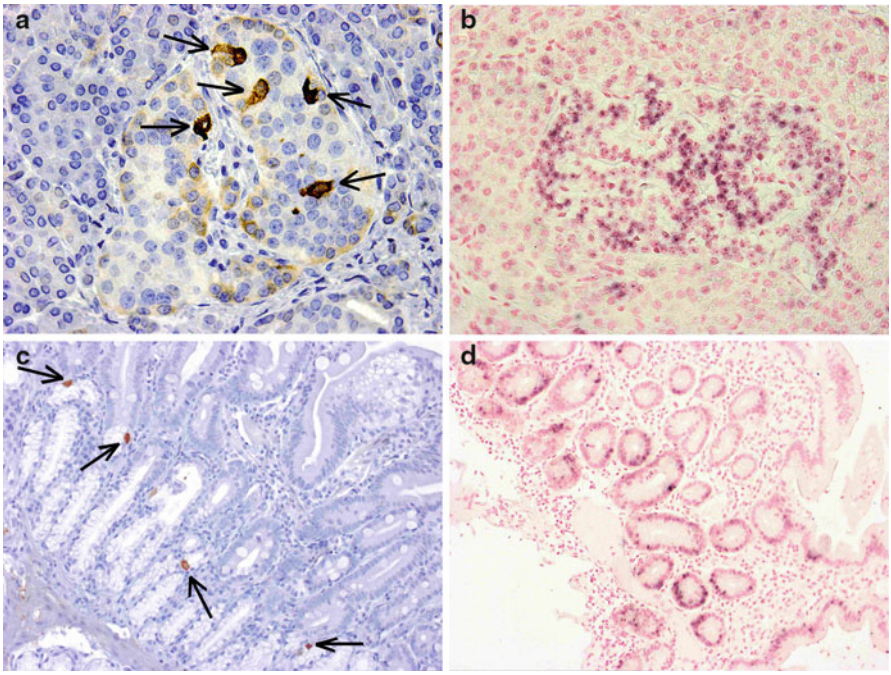

Fig. 13.1 Detection of enterovirus in the pancreas (**a**, **b**) and small intestinal mucosa (**c**, **d**). In panels **a** and **c** the *brown color* indicates the presence of enterovirus VP1 protein (immunohistochemistry) and in panels **b** and **d** the *dark purple* precipitate indicates the presence of enteroviral genome (in situ hybridization). Pancreas samples were provided by nPOD and small intestinal mucosa samples by Professor Markku Mäki

samples and usually needs frozen or fresh tissue or samples which have been treated with special RNA preservation buffers. IHC is based on specific antibodies against enterovirus proteins, whereas ISH uses probes designed to hybridize with the enteroviral genome. Pancreatic tissue samples are usually obtained from autopsy or organ donation, although pancreas biopsies have also been performed occasionally (Imagawa et al. 2001).

IHC and ISH have been developed and optimized to be used for both formalin-fixed paraffin-embedded and frozen samples. Unlike other enterovirus screening methods, IHC and ISH enable the localization of virus in different regions and anatomical sites of the target tissue and, using double-staining with specific antibodies against enterovirus and pancreatic islet hormones such as insulin, glucagon, somatostatin, they allow localization of the virus in different cell types.

The antibody used most frequently for detection of enterovirus by IHC is a commercial monoclonal antibody clone 5-D8/1 (DakoCytomation). This antibody was developed in 1987 (Yousef et al. 1987) and it recognizes a conserved group-specific epitope in enteroviral VP1 capsid protein (Samuelson et al. 1995). It reacts with a

wide range of different enterovirus serotypes in infected cell culture samples (Trabelsi et al. 1995; Oikarinen et al. 2010). On the other hand, it has been shown in some studies (Terletskaia-Ladwig et al. 2008; Miao et al. 2009) that this antibody fails to detect several CAV and echovirus serotypes, as well as EV68-71 serotypes. One important concern is that this clone has been reported to cross-react with certain host proteins such as HSP60/65 and IA-2 which are expressed in the pancreas (Harkonen et al. 2000, 2002). It may also react with uninfected human cardiomyocytes (Klingel et al. 2004), vascular smooth muscle cells, and centroacinar cells in the exocrine pancreas (Richardson et al. 2009). The Enterovirus Screening Set (Chemicon) includes four species-specific antibody blends (Coxsackievirus B Blend, Echovirus Blend, Enterovirus Blend and Poliovirus Blend, and Pan-Enterovirus Blend). The Pan-Enterovirus Blend is a mixture of two monoclonal antibodies 9D5 and 2E11, presumably designed against a virus-encoded, non-virion determinant (Yagi et al. 1992). These antibody mixtures have also been reported to react widely with different enterovirus serotypes and also with viruses other than enteroviruses (Miao et al. 2009). Thus, IHC has been used widely for detection of enterovirus proteins in tissue samples. However, caution is needed in the interpretation of positive findings since cross-reactivity with host antigens may occur. Optimally stringent assay conditions are critical for avoiding this cross-reactivity, and confirmation of positive staining with other methods is recommended.

Non-isotopic ISH applications have became more and more popular and have replaced isotopic methods. For example, digoxigenin-labeled probes are used commonly in enterovirus detection (Hohenadl et al. 1991; Oikarinen et al. 2010). The probes are generally designed to hybridize with a conserved sequence which is common to all known enterovirus serotypes, and also species-specific probes can be used (Foulis et al. 1997; Ylipaasto et al. 2004). ISH is technically quite challenging due to multiple assay steps, and it is always a compromise to obtain a sufficiently strong enough positive signal without gaining high levels of background staining. As in PCR, positive and negative controls should be included in each test run to monitor the quality and reproducibility of ISH assays.

It has been suggested that persistent enterovirus infection in the small intestine and/or in the pancreas may be an important factor in the pathogenesis of type 1 diabetes. Detection of this kind of slowly replicating persisting virus is much more challenging than that of actively replicating virus. During an acute enterovirus infection, the amount of positive-stranded RNA is 50–100 times higher than that of negative-stranded RNA (Chehadeh et al. 2000). In contrast, in persistent infection the amount of positive- and negative-stranded RNA is about equal and the synthesis of capsid proteins can be decreased. In this case virus replication occurs mainly at the RNA level (Klingel et al. 1992) where double-stranded viral RNA complexes are formed (Tam and Messner 1999). Antibodies against double-stranded RNA and PKR (dsRNA-dependent protein kinase) have been used to detect these molecules in cell and tissue samples (Richardson et al. 2009, 2010), which offers one option for detection of viral persistence. It is also possible to use strand-specific ISH or RT-PCR to study the balance between positive- and negative-stranded viral RNA (Klingel et al. 1992; Foulis et al. 1997; Ylipaasto et al. 2004).

The limited availability of samples from the pancreas of patients with type 1 diabetes has hindered the progress in this research field. The process leading to type 1 diabetes progress is usually slow and it would be vital to show the presence of the virus at the beginning of the process. This creates a huge challenge for studies with pancreatic tissue, since such samples should be taken long before type 1 diabetes is diagnosed. Another important aspect relates to the processing of tissue samples. Pancreatic enzymes start to degrade the tissue very rapidly; therefore, the sample should be either fixed or frozen immediately, and in case of autopsy samples, the post-mortem time should be as short as possible. Formalin-fixation is also known to degrade some of the RNA.

The diabetic process in the pancreas is often "patchy" (Foulis 1996), i.e., morphological changes can vary in different parts of the pancreas. Thus, a single sample from one part of the pancreas does not necessarily provide a representative view of the disease process. Possible enterovirus infection might be restricted to certain part of the organ and several sections of the pancreas should be examined to detect the virus using ISH or IHC.

In summary, the detection of enteroviruses in tissue samples is demanding and the results have been varied. Because of the reported cross-reactivity with host tissue and possible low sensitivity of some enterovirus antibodies, it is highly recommended to confirm the result of IHC using other methods. It is also important to take into consideration the possible viral persistence when interpreting the results. In persistent infections, the viral genome should be detectable using ISH, while synthesis of viral proteins may be at a very low level leading to negative result by IHC.

Antibody Assays

Virus antibodies are usually measured in serum or plasma, but other sample types such as whole blood, dried blood spots, and stool samples can also be used. The sensitivity of these assays for detection of enterovirus infections depends on several factors including the technical set-up of the assay and the sample type. Acute infections are typically diagnosed by the presence of virus-specific IgM in a single sample or by detection of increases in antibody levels (IgG, IgM, or IgA) between two serial samples. Usually, enterovirus antibodies are measured using enzyme immunoassay (EIA) and previously also by using radioimmunoassay. Infection history can be studied by measuring IgG class antibodies, but it should be noted that IgG responses which are detected by EIA can be transient lasting only for a few months. In longitudinal sample series, such as those collected in prospective follow-up studies, the length of sampling interval has a critical impact on assay sensitivity. Long sample intervals can lead easily to false-negative findings since transient antibody responses remain undetected. In contrast to antibodies measured by EIA, the antibodies measured by a neutralization assay last longer and can be used as a marker of past infection ("serological scar").

Serological diagnosis of enterovirus infections is more complex than that of many other virus infections. The reason for this is the large number of different enterovirus serotypes which makes it difficult to cover them all by antibody assays. EIA is the method used most widely. Its ability to detect enterovirus antibodies depends on the antigen which is used in the assay, immunoglobulin isotype measured, set-up of the assay, and the nature of sample material. It is important to realize that the antibodies which are detected by EIA are not usually specific for any particular enterovirus serotype, since enteroviruses contain antigenic structures which are common to several different enterovirus serotypes. These cross-reactive epitopes become exposed on the virus surface when it becomes attached to the plastic surface of the EIA plate (Torfason et al. 1988). In spite of this broadly reactive nature of enterovirus antigens, the EIA antibody assays do not cover all enterovirus serotypes. Thus, using a single serotype as an EIA antigen, it is not possible to detect antibodies against all enteroviruses, but rather a subgroup of antigens. Synthetic peptides carrying epitopes common to several enteroviruses have been successfully used as broadly reactive antigens in EIAs (Hovi and Roivainen 1993; Samuelson et al. 1994; Hyöty et al. 1995).

The standard indirect EIA where the virus antigen is bound to the plastic is usually suitable for the measurement of IgG and IgA class antibodies against enteroviruses. However, reliable measurement of IgM usually requires an antibody-capture format where IgM class immunoglobulins are first captured by anti-human IgM antibodies on EIA plate, followed by incubation of serum, virus, and detection layer, respectively. This type of capture assay can be also used for IgG and IgA measurements. The advantage of such antibody-capture assays is that they eliminate the competition between different antibody classes in the binding to the virus antigen.

The most sensitive and specific serological method is the measurement of neutralizing antibodies. By definition, these antibodies can neutralize the infectivity of the virus in vitro. Neutralizing antibodies do not usually cross-react between different enterovirus serotypes and can therefore identify the type of the virus causing the infection. These antibodies also remain elevated for several years (even decades) after the infection making it possible to study the past infection history. However, transient antibody responses also occur, and serological responses may even be absent, particularly if the titer of virus exposure has been small (Saliba et al. 1968). In addition, immune protection against enteroviruses is mainly based on neutralizing antibodies giving an important biological correlate for the antibody results. In spite of these important advantages, neutralizing antibody assays have not been widely used in studies evaluating enterovirus-diabetes association. This is due to the expensive and labor-intensive techniques (sterile cell culture work) as well as the required special knowledge of enterovirus biology which have hindered the extension of these methods outside specialized virus laboratories. In addition, the serotype-specific nature of these antibodies means that several assays must be carried out in parallel to measure antibodies against different enterovirus serotypes. Thus, large-scale screening of neutralizing antibodies against several enterovirus types is extremely more expensive compared to standard EIA techniques.

In conclusion, antibody assays have important advantages over direct virus detection methods. They make it possible to study past enterovirus exposures (serological scar) and diagnose acute infections from samples which have been taken after the infection has been cleared and the virus is no longer detectable. The combined use of direct virus detection and virus serology can considerably increase the sensitivity of enterovirus diagnosis in studies evaluating the viral etiology of type 1 diabetes.

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