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Evaluation of immunohistochemistry and *in situ* hybridization methods for the detection of enteroviruses using infected cell culture samples

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ABSTRACT

Background: Enterovirus infections are frequent in all age groups. In addition to acute infections, they have been connected to chronic diseases such as cardiomyopathies and type 1 diabetes. Based on this there is an increasing need for the reliable detection of enteroviruses in different kinds of tissue samples.
Objectives: The aim of this study was to set up a test panel which can detect a wide range of different enteroviruses in paraffin-embedded samples and fresh frozen samples using immunohistochemical and *in situ* hybridization methods.
Study design: A panel of nine enterovirus antibodies was optimized for the detection of different enterovirus types in both paraffin-embedded and frozen cell culture samples. In addition, an oligonucleotide probe detecting all human enteroviruses was evaluated for ISH in formalin-fixed paraffin-embedded cell culture samples.
Results: Most antibodies worked well in both sample types. Some antibodies detected only one of the tested serotypes, whereas others detected several serotypes. ISH was able to detect all tested enterovirus types.
Conclusions: This test panel makes it possible to detect a wide range of different enterovirus types in both formalin-fixed paraffin-embedded and frozen samples. The same methods can also be applied for tissue sections, but may need further optimization for each tissue type.
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1. Background

So far already over 100 different human enterovirus types have been identified. They cause usually mild symptoms but are also a significant cause of more severe diseases such as myocarditis, meningitis, encephalitis, systemic infections in newborns and paralysis (e.g. polio myelitis). Enterovirus infections may also play a role in chronic diseases such as dilated cardiomyopathy,[1–3] chronic fatigue syndrome[4] and type 1 diabetes.[5] Enteroviruses have been found in heart tissue in chronic cardiomyopathies and in the pancreas and intestine of type 1 diabetic patients using immunohistochemistry (IHC), *in situ* hybridization (ISH), RT-PCR and virus isolation.[6–10]

The detection of enteroviruses in tissue samples is technically challenging, especially if only formalin-fixed samples are available, as virus isolation is not possible from such samples and PCR-based methods may not work optimally due to the degradation of viral RNA. Although there are some studies showing the amplification of viral RNA in archived paraffin-embedded samples using RT-PCR,[11–13] other methods, such as *in situ* hybridization and immunohistochemistry, are also essential in order to localize the virus and to understand viral pathogenesis. In addition, the large number of enterovirus serotypes makes it difficult to cover them all using a single oligonucleotide probe or enterovirus-specific antibody. The low amount of virus and possible presence of PCR inhibitors in tissue samples create an additional challenge for sensitivity.

Abbreviations: ATCC, American Type Culture Collection; CAV, coxsackie A virus; CBV, coxsackie B virus; CMV, cytomegalovirus; DAB, diaminobenzidine; GMK, green monkey kidney; HPeV, human parechovirus; IHC, immunohistochemistry; ISH, *in situ* hybridization; nPOD, Network for Pancreatic Organ donors with diabetes; PV, poliovirus.

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Most immunohistochemical studies have been based on a single enterovirus-specific antibody.[3,7,14] Even though some of these antibodies target to epitopes which are common for several enterovirus serotypes, it is not clear how well they can cover different enterovirus types in tissue samples. The specificity of positive staining with one antibody should also be confirmed using another antibody or alternative methods.[9] Due to the increasing evidence suggesting that enteroviruses play a role in the pathogenesis of chronic diseases there is a need to develop validated tests which can be used to detect enteroviruses in various kinds of tissue samples.

2. Objectives

The aim of this study was to develop a test panel, which includes both immunohistochemical stainings and *in situ* hybridization and which allows reliable detection of enteroviruses in samples prepared with different methods. The methods were evaluated using infected cell culture samples, which were either frozen or fixed in formalin and embedded in paraffin.

3. Study design

3.1. Infected control cell sections

Green monkey kidney cells (GMK and Vero), vervet monkey kidney cells (MA104), human fibroblast cells (HEL-7), carcinomic human cervix epithelial cells (HeLa) and carcinomic human alveolar basal epithelial (A549) cells were grown to a monolayer. GMK cells were infected with coxsackie B virus (CBV1-6), coxsackie A9 virus, echovirus (echovirus 3, 6, 9 or 11), enterovirus 71 or poliovirus type 3 (PV3). Vero cells were infected with coxsackie A16 virus, A549 cells with echovirus 30 or human parechovirus 1 (HPeV1), MA104 cells with rotavirus, HeLa cells with adenovirus, and HEL-7 cells with cytomegalovirus (CMV). Infected cells were either frozen in liquid nitrogen or fixed in formalin for 24 h and embedded in paraffin. 5 μm cryostat or paraffin sections were cut onto Superfrost Plus microscopic slides (Menzel-Glaser, Braunschweig, Germany). The cells and viruses were obtained from the American Type Culture Collection (ATCC), except the serotype echovirus 3, which was isolated in our laboratory.[15]

3.2. Antibodies

Nine different antibody reagents were used, of which six were commercial mouse antibodies or antibody blends, and three were in-house rabbit polyclonal antibodies. Pan-Enterovirus Blend, Enterovirus Blend, Coxsackievirus B Blend, Echovirus Blend and Poliovirus Blend were purchased from Chemicon (Temecula, CA) and enterovirus mouse monoclonal antibody (MAb 5-D8/1) from Dako Cytomation (Glostrup, Denmark). Rabbit polyclonal antibodies against CBV3, CAV16 and echovirus 11 were in-house antibodies produced as previously described.[16]

3.3. Immunohistochemical staining

Frozen samples were fixed onto microscopic slides by air-drying overnight. Formalin samples were placed in 10% formalin for 24 h and routinely processed and embedded in paraffin. Antigen retrieval was performed on re-hydrated sections in a microwave oven at 850 W for two 7-min cycles using Tris–EDTA buffer (pH 9.0) as the retrieval solution. Immunostaining of both frozen and paraffin samples was performed manually using EnVision^{TM} polymer technique (DakoCytomation). Slides were rinsed between staining steps with 0.05 M Tris–HCl + 0.1% Tween 20 (pH 7.2) for 3× 5 min. Diaminobenzidine (DAB) was used as chromogen and haematoxylin as nuclear stain. Immunostaining with echovirus 11, CAV16 and CBV3 in-house antibodies was performed as previously described.[8] The specificity of immunohistochemistry was controlled by omitting or replacing the primary antibodies with irrelevant antisera and using mock-infected cells or cells infected with other viruses than enteroviruses as negative control.

3.4. In situ hybridization

The presence of enteroviral genome in paraffin-embedded sections was analyzed using *in situ* hybridization as described in our previous studies.[7,8]

4. Results

4.1. Optimization of immunohistochemical staining for frozen sections

Pan-Enterovirus Blend, Coxsackievirus B Blend, Echovirus Blend, Poliovirus Blend, enterovirus antibody (5-D8/1) and in-house CBV3 and echovirus 11 antibodies were optimized for frozen sections using infected and uninfected cells and different fixation techniques, antibody dilutions and antibody incubation times. Fixation step was optimized by comparing virus-specific staining in infected and non-infected cells which were fixed by air-drying overnight at RT, in −4 °C acetone for 10 min, in −20 °C methanol for 3× 2 min or in frozen paraformaldehyde for 1.5 min. Air-drying worked well with all antibodies and was chosen for further experiments. At least four different antibody dilutions and three incubation times were tested for each antibody. The optimal incubation time was 30 min for every antibody. The optimal antibody dilutions were: enterovirus antibody (5-D8/1) 1:400, Pan-Enterovirus Blend 1:6, Coxsackievirus B Blend 1:2, Echovirus Blend 1:4, Poliovirus Blend 1:2, in-house CBV3 1:200 and in-house echovirus 11 1:100.

4.2. Optimization of immunohistochemical staining for paraffin sections

All nine antibody blends or antibodies were optimized for formalin-fixed paraffin-embedded sections using infected and uninfected cells and different antibody dilutions. Optimal antibody dilutions were: enterovirus antibody (5-D8/1) 1:1000, Pan-Enterovirus Blend 1:2, Coxsackievirus B Blend 1:1, Echovirus Blend 1:2, Poliovirus Blend 1:1, in-house CBV3 1:200, in-house CAV16 1:100 and in-house echovirus 11 1:100. It was not possible to determine an optimal antibody dilution for Enterovirus Blend, as it gave similar staining in both infected and uninfected cells in all tested assay conditions. This antibody was not used in further analyses described below.

4.3. Detection of enteroviruses by different assays

Each antibody was tested for its ability to detect different enterovirus serotypes in frozen (Table 1) and formalin-fixed paraffin-embedded (Table 2) cells infected with different enterovirus serotypes. Most antibodies worked well binding to at least one enterovirus serotype in both frozen and paraffin-embedded samples. However, one of the antibodies, Coxsackievirus B Blend, worked only with frozen samples. Altogether, frozen sections were more often positive and showed more intense staining with most of the antibodies. Some antibodies were able to detect only one of the tested serotypes (Poliovirus Blend and in-house echovirus 11 and CAV16 antibodies), whereas others detected several serotypes. For example, Coxsackievirus B Blend was specific for CBV1–CBV6 in frozen samples but did not stain formalin-fixed samples at all. Thus, this antibody was able to distinguish CBV serotypes

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