Synthetic peptides for efficient discrimination of anti-enterovirus antibodies at the serotype level

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A R T I C L E   I N F O

Article history:
Received 4 January 2014
Received in revised form 22 April 2014
Accepted 23 April 2014
Available online 11 June 2014

Keywords:
Enterovirus
Coxackievirus
Serotypes
Homotypic antibodies
Heterotypic antibodies
VP1 protein

A B S T R A C T

Enteroviruses are important human pathogens, causing a broad spectrum of diseases from minor common colds to fatal myocarditis. However, certain disease syndromes are caused by one or few serotypes. Serotype identification is difficult due to the laborious neutralization tests that lack of sensitivity, while in commercial ELISAs homotypic antibodies' activities are largely masked by the recognition of genera-specific epitopes by heterotypic antibodies. In the present study homotypic assays were developed with the ability to discriminate different enterovirus serotypes. Seventy-three children sera, positive for IgM antibodies against enterovirus genus and 49 healthy children were examined for the presence of antibodies against 14 synthetic peptides derived from a non-conserved region of the VP1 protein of coxsackieviruses B2, B3, B4, B5, A9, A16, A24, echoviruses 6, 7, 9, 11, 30, enterovirus 71 and parechovirus 1. 50% of the anti-enterovirus IgM positive sera (>150 BU) reacted with the peptides with the majority of them to preferentially recognize one of them, supporting the homotypic nature of our assay. Inhibition studies yielded homologous inhibition rates 67–95% suggesting that specific peptide recognition actually occurred. The diagnostic value of our assay was tested in blood samples drawn over a 1.5-year period from a 5-year old patient. The anti-enterovirus reactivity was clearly attributed to echovirus serotype 11. The IgM/IgG antibody ratio was reversed 4 months later and subsequently IgM antibodies dropped below the cutoff point. In this paper we demonstrate that our assay can be used to discriminate between antibodies targeting different enterovirus serotypes.

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Introduction

Human enteroviruses are ubiquitous viruses found throughout the world [28]. The non-polio enteroviruses include group A and B coxsackieviruses, echoviruses and “newer” enteroviruses. Coxsackieviruses are divided based on their antigenic differences in two serogroups, A (CVA) and B (CVB), comprising 23 and 6 serotypes, respectively, while echoviruses include 32 serotypes and “newer” enteroviruses with 5 serotypes [1,28,39]. The non-polio enteroviruses are responsible for a wide spectrum of disease in humans of all ages. The majority of non-polio enteroviruses infections are asymptomatic [39,41] but in some cases, can lead to severe diseases, such as aseptic meningitis [6], pancreatitis [15,24], hepatitis [9,17,38] and myocarditis [18]. Although the disease spectra of different serotypes overlap considerably, some syndromes are caused by one or a few serotypes [27]. CVB3 is responsible for 63% of myocarditis cases and may lead to dilated cardiomyopathy [7,13,19]. CVB4 and CVB5 are associated with many cases of central neural diseases, while CVA24 has been associated with respiratory illness and acute hemorrhagic conjunctivitis. CVA16 is responsible for most cases of hand, foot and mouth disease (HFMD) [10]. Additionally, CVB4 has been implicated in the development type 1 diabetes and pancreatitis [12,16].

Laboratory diagnosis of enteroviral infection is laborious as it involves isolation and characterization of the causative virus. However, some enterovirus serotypes particularly in the coxsackievirus A group, do not grow in cell cultures [22]. In addition, the opportunity to isolate a virus from a clinical specimen in cell culture is low. Alternatively, diagnosis can be performed by the detection of neutralizing IgM antibodies that inhibit the cytopathic effect caused by the virus [27,33]. Despite its wide use, the serum neutralization test is relatively insensitive and has several limitations since it requires the use of live virus and cell cultures (if it is a
serotype that grows in cells) and is time-consuming. Molecular methods such as polymerase chain reaction (e.g. nested PCR) are also used for the detection of viral ribonucleic acid [8]. Although these tests possess increased sensitivities in CSF and blood specimens, their utility is limited to the viremia phase of the infection (usually over a few days). Nasopharyngeal secretions and feces have no diagnostic value since they are extremely liable to contamination. Enteroviral infection can also be documented by serological detection of antibodies using ELISA assays. These tests are sensitive, independent of cell cultures and do not require the isolated virus. In addition, they provide diagnostic information within a few hours and the experiments are relatively inexpensive to perform. However, the frequent cross-reactions among antigens from different enteroviruses (e.g. coxsackie, echo, enterovirus, etc.) limit the diagnostic value of ELISA [11,25,26,37]. In addition, the recognition of serotype-specific epitopes by homotypic antibodies is largely “masked” by the recognition of “genera-specific” cross-reactive epitopes by heterotypic antibodies, preventing the identification of specific viral serotypes.

In this study, we develop a homotypic immunosorbent assay (ELISA) which can discriminate between different enteroviruses and human parechoviruses serotypes. This assay is based on selected peptide epitopes from the non-conserved regions of VP1.

**Materials and methods**

**Sera**

73 child sera, positive for IgM antibodies against enterovirus genus (as assessed by a commercial anti-enterovirus assay, Serion Immunodiagnostics, Würzburg, Germany) were collected in the Panagiotis & Aglaia Kyriakou Children’s Hospital over a period of 2 years, 2011–2012. All children had symptomatic manifestations such as pleurodynia, symptoms of respiratory tract infection, exanthemas, and symptoms of CNS infection (aseptic meningitis). Unfortunately, detailed patient information was not available. 49 sera, negative for IgM antibodies against enterovirus, were obtained from healthy children in the same hospital. All sera were tested for the presence of IgG and IgM antibodies by enzyme-linked immunosorbent assay (ELISA) against synthetic peptides derived from coxsackieviruses B2, B3, B4, B5, A9, A16, A24, echoviruses Ech6, Ech7, Ech9, Ech11, Ech30, enterovirus 71 and parechovirus 1.

**Synthetic peptides**

Synthetic peptides derived from a non-conserved region of VP1 protein of coxsackieviruses B2, B3, B4, B5, A9, A16, A24, echoviruses 6, 7, 9, 11, 30, enterovirus 71 and parechovirus 1 (previously designated as echovirus 22) were synthesized using automated Fmoc (N-[9 fluorenlyl] methoxycarbonyl) solid-phase synthesis [2] (Bio-Synthesis, Lewisville, TX). The peptides were purified by high-pressure liquid chromatography (HPLC) and their identities and purities (>90%) were confirmed by mass spectroscopy (MS).

**ELISAs**

The reactivity of sera against enterovirus genus was investigated using a commercially available assay for IgM and IgG antibodies (Serion ELISA classic enterovirus; Serion Immunodiagnostics, Würzburg, Germany). The basis of the antigen preparation used in this assay is a mixture of heat-denatured coxsackievirus type B5 and echovirus 6 virions. This antigen preparation was reported to be widely cross-reactive, facilitating the detection of infections due to other enterovirus species and serotypes [36]. The assay was performed according to the manufacturers and extinction signals were converted to quantitative values of antibody concentrations using 4 parameter logistic-log-model (4 PL) curve fitting.

The evaluation of sera reactivity against synthetic peptides derived from 14 different enterovirus serotypes was performed using a custom ELISA assay. Briefly, high-binding microplates (Costar TM; Corning Life Sciences, Acton, MA) were coated with synthetic peptides in PBS, pH 7.2 at concentrations 2–10 μg/mL (optimal concentration was determined separately for each peptide in preliminary experiments), for 4 h at room temperature (RT). After blocking the free binding sites with 200 μL blocking buffer (2% BSA-PBS) for 1 h at RT, the wells were washed with PBS containing 0.05% Tween 20 (PBS-T). Sera were preincubated for 15 min with RF absorbent (1:100, Serion Immunodiagnostics, Würzburg, Germany) and thereafter added to the wells at 1:200 dilution factor in blocking buffer and incubated overnight at 4 °C. After three washes with PBS, alkaline phosphatase-conjugated anti-human IgG (Jackson ImmunoResearch, West Grove, PA), diluted 1:1000 in blocking buffer, was added and incubated for a period of 1 h at room temperature. The plates were washed with PBS-T and optical densities (ODs) were quantified at 405 nm with an ELISA reader (Bio-Tek Instruments, Winooski, VT). All ODs were transformed and expressed as binding units according to the following formula:

\[
\text{Binding units (BU)} = \left( \frac{\text{OD}_{\text{Sample}}}{\text{OD}_{\text{Cutoff}}} \right) \times 100
\]

where OD Sample is the OD reading of the current sample and OD Cutoff is the cutoff value. The cutoff value for anti-peptide ELISA was calculated as mean normal sera OD plus 5 standard deviations. All normal sera were obtained from healthy individuals and were selected to be negative for IgG and IgM antibodies in Serion classic enterovirus ELISA.

**Fig. 1.** Homology of amino acid sequences of synthetic peptides derived from VP1 protein from the viruses Coxackie B4 (CVB4pep), Coxackie B2 (CVB2pep), Coxackie B5 (CVB5pep), Coxackie B3 (CVB3pep), Echo 9 (EV9pep), Echo 6 (EV6pep), Echo 11 (EV11pep), Echo 7 (EV7pep), Coxackie A9 (CVA9pep), Echo 30 (EV30pep), Coxackie A16 (CVA16pep), Coxackie A24 (CVA24pep), Enterovirus 71 (ENT71pep) and Parecho 1 (PARV1pep). Different gray tones represent differences in sequence similarities.
Inhibition assay

In order to examine the specificity of peptide recognition by IgM antibodies, inhibition ELISA experiments were performed. Selected sera (#469, #1713, #4067, #2023, #264, #1144) were diluted at 1:300 and pre-incubated for 3 h at RT with different concentrations, ranging from 1 to 10 μg/mL, of selected peptides (pepCVA16, pepCVB5, pepPar1, pepEch9, pepEch11 and pepCVB2, respectively). They were, subsequently, examined by IgM-ELISA on plates coated with the homologous peptides as previously described. A peptide corresponding to amino acids 250–257 of Leishmania glycoprotein gp63 (IASRYDQL) was used as control.

Statistical analysis

Statistical analysis was performed using the Minitab v16.0 and GraphPad Prism v5.03 software. One-way analysis of variance (ANOVA) was used for statistical analysis of the anti-peptide reactivity in each serum, followed by the Tukey post hoc test for pair-wise comparison of samples. The significance of the difference in frequency of peptide-recognition among groups of different reactivity in genus-specific anti-enterovirus ELISA was determined with the Chi-square test. Mean titer of IgM anti-peptide antibodies between different groups was compared by using Student’s t test. A value of p < 0.05 was considered statistically significant.

Results

Design of the synthetic peptides

Most variable enteroviral proteins are structural proteins and among them VP1 possess the highest sequence variation [23,27,30]. Our sequence analysis of VP1 revealed that the major differences among enteroviral species and serotypes are found in the amino-terminal region of the VP1 antigen. This region has been previously found to hold major B cell antigenic determinants [3,14]. One antigenic determinant, representing the amino-terminal 1–15 residues of VP1 is a serotype specific epitope for coxsackievirus B3 [14]. This epitope is adjacent to a group–common, broadly cross-reactive epitope of enteroviruses, located in region 26–55 of VP1 [3,34,35]. In this regard, it is most likely that VP1 holds two adjacent antigenic determinants in its N-terminus, one targeted by homotypic antibodies and other targeted by heterotypic antibodies. In this study, peptides spanning amino-terminal region 1–15 of coxsackieviruses B2, B3, B4, B5, A9, A16, A24, echoviruses Ech6, Ech7, Ech9, Ech11, Ech30, enterovirus 71 and parechovirus 1 were synthesized (Fig. 1) with primary sequences representing the 10 most common enterovirus serotypes, submitted to the Centers for Disease Control and Prevention in USA between 1970–2005 and 2006–2008 [4,5]. Echoviruses Ech9, Ech11, Ech30, Ech6, Ech4, Ech7 and coxsackieviruses B5, B2, A9, B4, B3, B1 represent 77% of the total serotypes reported between 1970 and 2008. In addition, coxsackieviruses A16, A24, enterovirus 71 as well as parechovirus 1 (formerly known...
as echovirus 22) were included in our study, since they are correlated with certain clinical syndromes [21,41]. Peptides derived from VP1 of echovirus 4 and coxsackievirus B1 were not synthesized since their peptide sequences do not differ significantly from the other serotypes.

**Synthetic peptides derived from VP1 protein are able to discriminate IgM antibodies targeting different enterovirus serotypes**

73 positive and 49 negative sera for IgM antibodies against the enterovirus genus were used in this study. All sera were obtained from children hospitalized in Panagiotis & Aglaia Kyriakou Children’s Hospital during years 2011–2012. It was found that 42% of anti-enterovirus IgM positive sera had also IgG antibodies against the enterovirus genus (Fig. 2A). Thirty eight percent of them recognized one or more peptides in genotype-specific ELISA. However, the distribution of anti-peptide IgM positive samples followed a biased distribution toward the titer of enterovirus IgM antibodies in genotype-specific ELISA. More specifically, none of the borderline and weakly positive sera (100–150 BU, representing the 25% of total anti-enterovirus IgM positive sera) recognized any of the peptides in the IgM class assay (Fig. 2B). On the other hand, about 50% of sera with >150 BU of anti-enterovirus IgM antibodies reacted with at least one peptide in the same assay, exhibiting statistically significant higher reactivity than weakly positive sera (p < 0.01) (Fig. 2B).

Similar to antibodies of the IgM class, IgG anti-peptide antibodies were detected with statistically significant higher prevalence (p < 0.001) in sera producing high IgG titers in genotype-specific anti-enterovirus ELISA (Fig. 2C). Only 10% of sera with a negative or borderline reaction in commercial ELISA assay (0–150 BU) were positive for IgG anti-peptide antibodies, while 52% of highly positive sera (>150 BU) recognized at least one of the peptides in the IgG class assay.

Subsequently, the IgM anti-peptide activity was analyzed individually for each genotype-specific peptide. It was found that each synthetic peptide reacted with a proportion of the anti-enterovirus IgM positive sera (8–15%, depending on the peptide), with the peptides CVB5, CVB2 and Ech7 to be more commonly recognized (Fig. 3A). Sera-negative for anti-enterovirus IgM antibodies were used as controls. Only two of them exhibited a minor borderline reaction (100–110 BU) with one or more peptides (reactivity 0–2.0%, Fig. 3B). Anti-enterovirus IgM negative sera exhibited statistically lower reactivity in each anti-peptide ELISA than anti-enterovirus IgM positive sera (Fig. 3). When IgG anti-peptide activity was analyzed individually for each peptide, it was found that they reacted with 10.9%–34.2% of the anti-enterovirus IgM positive sera (Supplementary Fig. 1A) and 8.2%–26.5% of the anti-enterovirus IgM negative sera (Supplementary Figure 1B). No statistically significant differences were observed in anti-peptide reactivity of these two groups.

**Reactivity patterns of the sera against different genotype-specific peptides**

The majority of sera that were positive for IgM antibodies against the peptides preferentially recognized one of them (Fig. 4; sera #2857, #469, #1713, #4067, #1196, #2023, #264, #24, #2755) (p < 0.001, one-way ANOVA with Tukey’s post hoc test). This reactivity profile supports the homotypic nature of our ELISA assay. However, some minority sera (e.g. Fig. 4; sera #1444, and #4598) recognized more than one peptide suggesting that both homotypic and heterotypic recognition could have occurred. In these cases, the peptides reacted differentially, with less than the 30% of the synthetic peptides to be concurrently positives. ANOVA analysis indicated a further discrimination of positive reactivities (as indicated by * and # symbols in Fig. 4 and Supplementary Figure 2). These data suggest that even in these minority sera, our assay could provide information about the potential etiological agent(s) in the infection. We also noted some sera gave no significant reactivity with any of the peptides, while two sera samples (#4204, #3869) exhibited borderline and positive reaction, respectively, with all the synthetic peptides (Fig. 5). Given that the peptide sequences differed significantly (e.g. Fig. 1; PARV1pep, CVA16pep, CV4pep) this reaction could be considered as a false-positive (or false-borderline). In this regard, our assay provides additional control over the results obtained by the commercial anti-enterovirus ELISA. Representative reaction profiles for sera positive for anti-enterovirus IgM antibodies that did not give a significant reaction in any peptide-ELISA also are shown in Fig. 5.

Fig. 4. Anti-peptide positive sera: reactivity profiles of individual sera against all the synthetic peptides used in our study. Clear discrimination of IgM reactivity was observed in the majority of cases as indicated by ANOVA with post hoc Tukey test (F, p values are presented below each graph) Symbols: * and # indicate the dissimilar reactivities.

Ech9, Ech11, Ech30), enterovirus (peptide: ENT71), parecho (peptide: PAR1). The data obtained for 12 representative IgM positive sera are presented in Supplementary Figure 3. These assays could discriminate the IgM anti-enterovirus reactivity to species specific reactivity for the majority of sera that were positive for IgM antibodies against the peptides.

Assessing the specificity of peptide recognition

The specificity of peptide recognition of peptides was evaluated using inhibition assays. Homologous peptide (at a concentration of 10 µg/ml) inhibited the binding of sera by 67–95% (Fig. 6). On the other hand, the control peptide failed to significantly inhibit the peptide-IgM antibody interaction (inhibition <20% at 10 µg/ml) (Fig. 6).

Clinical application of the assay

A 5-year old girl was admitted with acute respiratory tract infection with fever (<39.3 °C), headache, catarrh, myalgias, and fatigue. Laboratory evaluation revealed a viral infection. Serologic evaluation was performed for antibodies against different infectious agents (Mycoplasma pneumonia, Enterovirus, Cytomegalovirus, Epstein Barr virus, Adenovirus, Influenza-A, Influenza-B and Parainfluenza virus) and revealed positive IgM and IgG antibodies against enterovirus. Since the detection of the latter was based on
genus-specific assay, we applied our peptide-ELISA in order to identify the exact serotype. We found that the patient’s serum preferentially recognized Ech11-peptide both in IgM and IgG ELISA. Notably, the OD produced in IgM ELISA was greater than that in IgG ELISA. The patient was followed up for about 1.5 years and during this period 4 more serum samples were obtained. It was found that 4 months after the initial sample there was a reverse in the IgM/IgG OD ratio in ELISA, with IgG reactivity exceeding IgM reactivity, suggesting that IgG seroconversion had already occurred (Supplementary Figure 4). The IgM reactivity was further reduced and dropped below the cut-off point three months later. Subsequent serum samples were found negative for IgM antibodies against Ech11-peptide. On the other hand, IgG antibodies was reduced but remained positive until the last sample studied (1.5 years after the initial sample). The heterotypic interference in this assay was minimal, recognizing the CVB2 peptide (in a much lesser extent than Ech11 peptide) in the initial sample. These data suggest the successful identification of the etiological agent in a child infection using the peptide-ELISA developed in this study.

Discussion

Enteroviruses are clinically important human pathogens, causing a broad spectrum of diseases from common colds to potentially fatal myocarditis and neurological disorders [18,21,40]. It is well known that specific enterovirus serotypes are linked to specific clinical disorders [20,28,29,32,40]. Diagnosis of these disorders depends mainly on virus isolation and detection in the viremia phase of infection or on characterization of the host immune response. The latter is measured either by the laborious virus neutralization test or by different commercial serological assays. The main drawback of these assays is that they rely on antigen preparations from heat-denatured virions, with epitopes that are broadly cross-reactive among various picornaviruses genera, species and serotypes. Therefore, the recognition of serotype-specific epitopes by homotypic antibodies is largely “masked” by the recognition of genera-specific cross-reactive epitopes by heterotypic antibodies, rendering the identification of the serotype of the infectious agent impossible. The present study aimed at the development of homotypic ELISAs which can discriminate different enterovirus serotypes. In this regard we constructed peptides derived from a highly antigenic region of the VP1 protein, which can differentiate between various enterovirus serotypes and species.

The peptides were tested on children sera, positive or negative for anti-enterovirus IgM antibodies (as assessed by a commercial genus-specific ELISA assay). Children were selected instead of adult patients because (i) they have uncomplicated serological profiles (e.g. fewer interfering IgG anti-enterovirus antibodies that could interfere in our assays) and (ii) enterovirus infections occur mainly in children. Indeed the enterovirus surveillance in USA for the period 1970–2005 revealed that approximately 60% of enterovirus detections were from children aged <4 years old and only 17% were from adults (>20 years old) [4].
We first noticed that borderline (cutoff: 100 BU) and weakly positive sera (100–150 BU) in commercial anti-enterovirus assay did not react with the peptides and have a low prevalence of IgG anti-enterovirus antibodies (corresponding to that observed for young children). These sera most probably are false positives or sera with very low affinity IgM antibodies (due to cross-reactions), which cannot be further discriminated. Our data suggest that the commercial assay's value is limited to sera exhibiting high-reactivity. About 30% of the sera with >150 BU of IgM antibodies in commercial anti-enterovirus ELISA recognized one or more peptides in our ELISA assay. The majority of them preferentially reacted with one single peptide, supporting the homotypical nature of our assay. Peptide specificity was further confirmed with inhibition assays whereas homologous peptides produced inhibition rates up to 95%. However, a minority of sera reacted with more than one peptide but ≤4 of total peptides, indicating that besides homotypic recognition, heterotypic reaction can also occur. In these cases, our assay provides more information than the commercial ELISA about the etiological agent(s). As the positive for anti-enterovirus IgM antibodies sera that did not give a significant reaction with any of the peptides are concerned, these sera most likely represent: (a) sera with low titer of anti-enterovirus IgM antibodies (25% of the total), which are either false positives or contain low-affinity antibodies, (b) sera that recognize other serotypes or epitopes not included in our synthetic peptides and (c) sera with antibodies against other picornaviruses that cross-reacted with enteroviral antigens.

Experiments also performed using species specific equimolar peptide mixtures for Coxsackie A, Coxsackie B, echo virus, enterovirus, parecho virus. These assays exhibited a better discrimination of reactivities than the ELISA using single peptides and therefore they can be used for species-specific identification of an enterovirus infection. However, the clinical correlations in the literature are rather serotype-specific than species-specific e.g. CVB3 is related to myocarditis while CVB4 is related to type 1
diabetes, CVA24 is related with acute hemorrhagic conjunctivitis while CVA16 is related to foot and mouth disease. In this regard, single peptide ELISAs can provide additional clinical information than species-specific ELISAs. However, the exact diagnostic value of the two types of the assays has to be evaluated in subsequent studies.

Serum IgM antibody against enteroviruses can often be detected early in the course of illness. Pozzetto et al. reported that 24% of patients with symptoms consistent with Enterovirus infection had IgM positive anti-enterovirus antibodies during early stage of infection, typically within 5 days [31]. Since clinical symptoms commonly occur after an incubation period of 3–5 days and a typical hospital stay spans more than 5 days, there is sufficient time to develop IgM antibodies in majority of cases. However, the same authors stated that their positive test results were not serotype specific and were of limited value due to cross-reactivities [31], a major pitfall of heterotypic assays. Since our assays produce minimal cross-reactions, we examined the diagnostic value of our assay in blood samples drawn over a 1.5 year period from a 5-year-old girl, who admitted to the Hospital with signs of a viral infection. Her serum was found positive for IgG and IgM antibodies against enterovirus and our assay successfully discriminated this reactivity to reactivity against echovirus 11. The validity of the detection was verified by the reversal of IgM/IgG reactivity ratio (observed 4 months later) followed by the reduction of IgM antibodies below the cutoff level (7 months later). Echovirus 11 is a common enterovirus that has been ranked second in frequency of reported enteroviruses in USA between 1970 and 2005 [4]. Our assay can be applied in clinical cases to provide valuable information for a more accurate diagnosis of picornavirus infections including the differentiation of enteroviral serotypes.

In conclusion, we successfully developed a novel ELISA capable of discriminating antibodies targeting different enterovirus serotypes. This assay could characterize host immune response against different enteroviral serotypes by distinguishing IgM and IgG reactivity and to monitor IgG seroconversion in serial samples.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2014.04.017.

References