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Short communication

Detection of intrathecal antibodies to diagnose enterovirus infections of the central nervous system

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ABSTRACT

Background: Enterovirus-D68 (EV-D68) predominantly causes respiratory disease. However, EV-D68 infections also have been associated with central nervous system (CNS) complications, most specifically acute flaccid myelitis (AFM). Diagnosing EV-D68-associated CNS disease is challenging since viral RNA is rarely detected in cerebrospinal fluid (CSF).

Objective: In order to determine an EV antibody index (AI), we evaluated the value of a commercially available quantitative ELISA to detect EV-specific antibodies in paired CSF and blood.

Study design: Nine paired CSF and blood samples were obtained from patients with EV-D68-associated AFM or from patients with a confirmed EV-associated CNS disease. EV-specific antibodies were detected using a quantitative ELISA. A Reiber diagram analysis was performed, by which the AI was calculated. Subsequently, EV ELISA results were compared with an EV-D68 virus neutralization test.

Results: ELISA detected EV-specific antibodies in 1 out of the 3 patients with EV-D68-associated AFM and in 3 out of the 6 patients with confirmed EV-associated CNS disease. In these patients, the AI was indicative for intrathecal antibody production against enterovirus. Assay comparison showed that EV-D68 neutralizing antibody detection increased the sensitivity of EV-D68 antibody detection.

Conclusions: A quantitative EV IgG ELISA in combination with Reiber diagram analysis and AI-calculation can be used as a diagnostic tool for EV-associated CNS disease, including EV-D68. An EV-D68 specific ELISA will improve the sensitivity of the tool. With the growing awareness that the detection of non-polio enteroviruses needs to be improved, diagnostic laboratories should consider implementation of EV serology.

Abbreviations

AI	antibody index
EV	enteroviruses
acute flaccid myelitis	AFM
cerebrospinal fluid	CSF
HHV6	human herpesvirus type 6
HSV1	herpes simplex virus type 1
HSV2	herpes simplex virus type 2
JC virus	John Cunningham virus
VZV	varicella zoster virus
EBV	Epstein-Barr virus
CMV	cytomegalovirus

1. Background

Several enteroviruses (EVs), *e.g.,* Coxsackievirus, Echovirus, EV-A71 and EV-D68, have been associated with central nervous system (CNS) diseases [1]. In 2014, EV-D68 caused outbreaks which were associated with respiratory diseases and neurological complications, including

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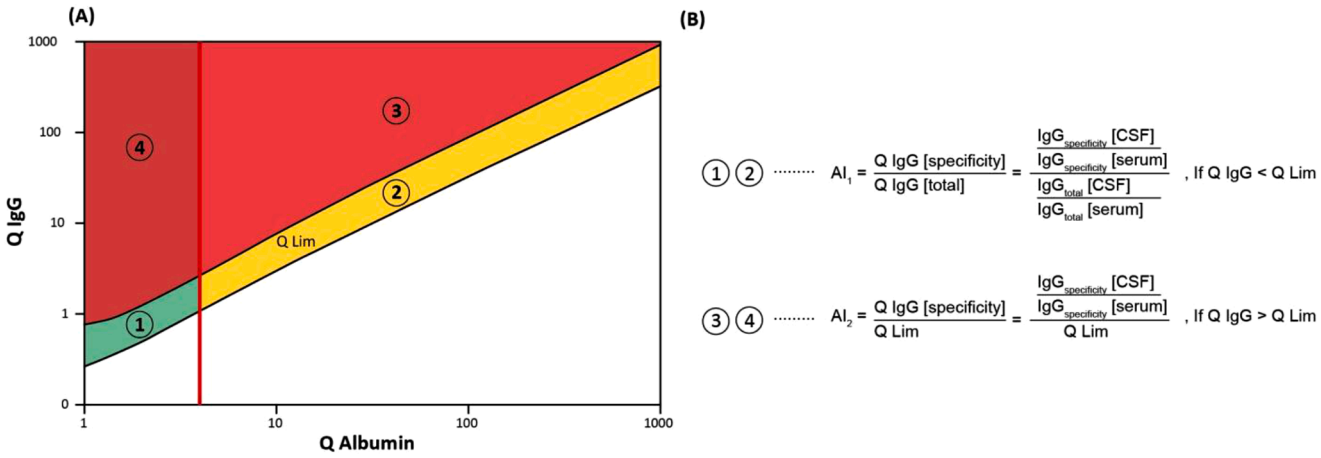

Fig. 1. Reiber diagram and antibody index. (A) Reiber diagram illustrates intrathecal IgG synthesis by presenting the IgG CSF-serum quotient (Q IgG) in relation to the albumin CSF-serum quotient (Q albumin), and showing the hyperbolic function with discrimination line (Q Lim) that indicates the upper reference range of Q IgG. The age-dependency for Q Albumin and CSF protein concentration is indicated by the vertical red line [16,17]. The diagram depicts 4 ranges: 1) Normal IgG, normal blood-CSF barrier; 2) Normal IgG, blood-CSF barrier dysfunction; 3) Intrathecal IgG synthesis, blood-CSF barrier dysfunction; 4) Intrathecal IgG synthesis, normal blood-CSF barrier. (B) Formula to determine the antibody index (AI) depends on the location of Q IgG within the diagram.

acute flaccid myelitis (AFM) [2,3]. Since then, EV-D68 causes biennial outbreaks, to a minimal extent in 2020, probably related to COVID-19-related measures [4,5]. Diagnostics to confirm EV-D68-associated CNS complications are challenging, since viral RNA is rarely detected in cerebrospinal fluid (CSF) [6]. In contrast, EV-D68-specific antibodies have been detected in the CSF of patients with EV-D68-associated CNS complications, which suggests viral invasion. Therefore, the European Non-Polio Enterovirus Network (ENPEN) has recommended to explore reliable detection of intrathecal antibodies against EVs. For this purpose, it is of relevance to study the widely available diagnostic tools [7–12].

When measuring virus-specific antibodies in CSF, it is essential to discriminate between those that are blood-derived and those that are synthesized locally in the CNS [13–15]. During CNS inflammation, the blood-CSF barrier function may be impaired, resulting in leakage of systemic antibodies into the CSF. In addition, decreased CSF flow, or polyclonal antibody expansion in the CNS, can affect the interpretation of CSF serology [16,17]. By using a Reiber diagram these potential influences can be corrected, increasing the reliability of the antibody index (AI) (Fig. 1) [15–18].

2. Objective

In paired CSF and blood samples from patients with EV-D68-associated AFM, a commercially available, quantitative EV ELISA was evaluated for its value to detect intrathecal antibodies against EVs. To assess the sensitivity of the EV ELISA in detecting EV-D68 specific antibodies, blood samples were tested both with ELISA and an EV-D68 virus neutralization test (VNT).

3. Study design

3.1. Specimen

Paired CSF and blood samples were collected from 3 patients with clinical signs of AFM. EV-D68 RNA was detected in their respiratory samples (EV-D68-AFM-#1-3). Based on clinical signs, patient history and the detection of EV-D68 RNA, these patients were considered as confirmed EV-D68 AFM. In comparison, paired CSF and blood (serum or plasma) samples from 6 patients with confirmed EV-associated CNS disease (EV-CNS-#1-6) were included. To determine the EV-ELISA specificity, 9 paired CSF and blood samples from patients with confirmed non-EV viral encephalitis (NON-EV-CNS) were included (HHV6, HSV1, HSV2, JC virus, parechovirus, VZV, EBV, CMV). Furthermore, blood samples from 4 patients with EV-D68 respiratory diseases were included (EV-D68-RTI-#1-4). Diagnostic specimens were provided by Erasmus MC, Rotterdam and Reinier Haga Medisch Diagnostisch Centrum, Delft, both in the Netherlands.

3.2. Serology

EV-specific antibodies were detected with a quantitative ELISA (SERION classic EV IgA, IgG and IgM) according to the manufacturer's protocol (Viron/Serion; Wurzburg, Germany). This CE marked, quantitative ELISA EV IgG has been validated for the detection of intrathecal antibodies in CSF. To assess the EV ELISA sensitivity, we compared the results with those of an EV-D68 specific VNT using blood samples from patients EV-D68-AFM-#2-3 and EV-D68-RTI-#1-4. The microneutralization assay was performed with two-fold sample dilution series which were incubated with EV-D68 subclade B3 (Genbank reference MN954541) (100 CCID50/ 60 ul per well) at 37°C for 1 hour. Next, rhabdomyosarcroma cells (ATCC) in Dulbecco MEM Eagle Medium (Lonza, Basel, Switzerland) supplemented with 1% (V/V) penicillin/streptomycin (Lonza), 1% (V/V) L-Glutamine (Lonza) and 10% (V/V) fetal bovine serum (Lonza) were added to the serum/virus-mix, and incubated at 33°C with 5% CO_2. The cytopathic effect was scored at day 5 post-inoculation. Based on assay validation, the cut-off value for positive VNT titer was >1/24.

3.3. Reiber diagram analysis and AI calculation

Albumin and total IgG in blood and CSF were measured by nephelometry. The age-dependent albumin CSF-blood quotient and CSF-blood immunoglobulin quotient were determined and analyzed using a Reiber diagram to correct for local synthesis of polyclonal IgG in the CNS. The AI is the ratio between CSF-blood quotient of the virus-specific IgG and total IgG, following Reiber's formula's (Fig. 1), with a cut-off value of 1.5 [15–19].

3.4. Ethical approval

Ethical approval was obtained from the Erasmus MC Medical Ethics Committee (MEC-2015-306) to anonymously analyze samples of included patients.

Table 1
Antibody Index (AI) determination in patients with confirmed EV-associated CNS infection or AFM and EV-D68 infections using SERION ELISA classic EV IgG.

Patient No.	Age	Diagnosis	Clinical specimen for diagnostic EV-PCR	Genotype	Blood sampling (days post diagnostic PCR)	EV ELISA blood IgG (U/ml)	Blood Total IgG (g/ml)	Albumin (g/ml)	EV ELISA CSF IgG(U/ml)	CSF Total IgG (g/ml)	Albumin (g/ml)	Antibody Index	Reiber diagram area
EV-D68-AFM-#1	2y	AFM	nasal wash	EV-D68	0	71.2	7.6	38.8	103.1	0.03	0.2	2.6	2
EV-D68-AFM-#2	1y	AFM	nasal wash	EV-D68	0	<5	n.d	n.d	<5	n.d	n.d	n.d	n.d
EV-D68-AFM-#3	4y	AFM	nasal wash	EV-D68	n.d.	8.8	n.d	n.d	<5	n.d	n.d	n.d	n.d
EV-CNS-#1	2mo	meningitis	CSF	CV-B5	0	<5	n.d.	n.d.	<5	n.d.	n.d.	n.d	n.d
EV-CNS-#2	37y	meningitis	CSF	CV-B5	0	41.6	12.3	46.1	46.8	0.05	0.5	0.9	2
EV-CNS-#3	30y	meningitis	CSF	E-5	1	20.4	10.8	47	6.7	0.03	0.2	1.5	4
EV-CNS-#4	30y	meningitis	CSF	E-6#	0	55.7	13.9	64	n.d.	0.03	0.2	2.7	4
EV-CNS-#5	42y	CSF tumor	CSF	n.d.	2	16	7.3	49	12.8	0.03	0.3	2.8	4
EV-CNS-#6	34y	meningitis	CSF	E-9	0	42.7	10.8	53.1	49.2	<0.003*	<0.09*	n.d	n.d

Positive (Pos) IgG cut-off is >15.0 U/ml. Equivalent (Eq) IgG is 11.0-15.0 U/ml. Negative (Neg) IgG cut-off is <11.0 U/ml. Y, years old; Mo, months old; CV, Coxsackievirus; E, Echovirus; EV-D68, Enterovirus D68; CSF, cerebrospinal fluid; AFM, acute flaccid myelitis; n.d, not detectable; # determined on feces
* below limit of detection (LOD). Cut-off value of AI > 1.5.

Table 2
Comparison of SERION ELISA classic EV IgA, IgM, IgG and virus neutralization test (VNT) in blood samples from patients with EV-D68-positive respiratory samples

Patient No.	Age	Diagnosis	Clinical specimen for diagnostic EV-PCR	Blood sampling (days post diagnostic PCR)	EV ELISA blood IgA	IgA (U/ml)	IgM	IgM (U/ml)	IgG	IgG (U/ml)	VNT titer
EV-D68-AFM-#2	1y	AFM	nasal wash	0	Neg	<4	Neg	<5	Neg	<5	240
EV-D68-AFM-#3	4y	AFM	nasal wash	0	Neg	5.9	Neg	<5	Neg	8.8	768
EV-D68-RTI-#1	50y	RTI	BAL	0	Neg	<5	Pos	22.2	Neg	5.5	768
EV-D68-RTI-#2	61y	RTI	throat swab	0	Neg	<4	Neg	<5	Neg	<5	48
				14	Neg	<4	Neg	<5	Neg	<5	48
				28	Neg	<4	Neg	<5	Neg	<5	96
				41	Neg	<4	Neg	<5	Neg	<5	192
				98	Neg	<4	Neg	<5	Neg	<5	288
EV-D68-RTI-#3	30y	RTI	throat swab	0	Pos	39.5	Pos	15.3	Eq	14.9	288
EV-D68-RTI-#4	61y	RTI	throat swab	6	Neg	4.8	Neg	<5	Neg	5.2	768
				38	Pos	15.1	Neg	<5	Pos	15.7	≥49152
				76	Neg	7.8	Neg	<5	Pos	17.7	≥49152
				93	Eq	10.3	Neg	<5	Eq	14.7	≥49152
				99	Neg	7.7	Neg	<5	Pos	18.1	36864

Positive (Pos) IgG cut-off is >15.0 U/ml. Equivalent (Eq) IgG is 11.0-15.0 U/ml. Negative (Neg) IgG cut-off is <11.0 U/ml. Y, years old; AFM, acute flaccid myelitis; RTI, respiratory tract infection; BAL, bronchoalveolar lavage; EV, enterovirus; VNT, virus neutralization test

4. Results

In 1 out of the 3 patients with EV-D68-associated AFM, antibodies above the assay cut-off were detected in CSF, and the EV IgG-AI was indicative for intrathecal antibody production against EV (EV-D68-AFM-#1). In patient EV-D68-AFM-#3, EV-specific antibodies were below the assay cut-off, while in patient EV-D68-AFM-#2 EV-specific antibodies were not detected (Table 1); therefore, an AI was not calculated. In patients with EV-confirmed CNS disease, a positive IgG-AI was determined in 3 out of the 6 patients (EV-CNS-#3-5). Altogether, 4 out of the 9 patients with confirmed or suspected EV-associated CNS disease had an AI above 1.5, which is suggestive for EV-specific intrathecal antibody production. The Reiber diagram analysis from all AI-positive patients supported intrathecal antibody production. Use of a corrected formula (depicted in Fig. 1, for range 3 and 4) was required based on evidence for polyclonal antibody production in 3 patients (range 4; EV-CNS-#3-5) (Table 1). Non-EV viral encephalitis CSF and blood samples all tested negative in the EV ELISA **(Supplementary table 1)**.

To determine the sensitivity of the EV ELISA, blood samples from patients EV-D68-AFM-#2-3 and patients EV-D68-RTI-#1-4 were tested by an in-house VNT. Overall, EV-D68 neutralizing antibodies were detected more frequently than were EV-specific IgG, IgA or IgM detected by ELISA. In patients EV-D68-AFM-#2-3 and EV-D68-RTI-#2, EV-specific Ig were not detected by the EV ELISA assay, while EV-D68 neutralization antibodies were. In patients EV-D68-RTI-#2 and #4, EV-D68 neutralizing titers increased in time, but this trend was not observed in EV IgA, IgM or IgG titers measured by ELISA (Table 2).

5. Discussion

This study demonstrated that a quantitative EV IgG ELISA combined with a Reiber diagram analysis and AI-calculation can be a useful addition to the diagnostic toolbox when studying EV-D68 associated CNS disease. To increase the sensitivity of EV-D68 specific antibody detection, a specific EV-D68 IgG ELISA should be developed.

Diagnostics for virus-associated CNS complications are challenging, as viral RNA is rarely detected in CSF. Specifically, EV-D68 viral RNA is detected in the CSF of only 3% of AFM cases, which proportion is low compared to other EVs [20]. The detection of intrathecal production of virus-specific antibodies provides indirect evidence for EV infection in CNS [13–15], and is therefore a useful addition to diagnostics. In previous studies, EV-specific antibodies in the CSF of patients with AFM were detected with the use of a peptide microarray [13] or VirScan technique [14], but supporting analyses of blood-CSF barrier function were not included. The lack of such analyses makes it difficult to distinguish virus-specific antibodies found in CSF from, for example, aspecific antibodies derived from polyclonal B cell stimulation in the brain. A Reiber diagram can increase the reliability of the results, but does always require collection of paired CSF and blood samples.

Serum EV IgG, IgM and IgA detection can be used for diagnostics of a respiratory EV infection [21], although samples ideally need to be collected before the start of intravenous immunoglobulin treatment. At the same time, levels of IgG will increase during the course of disease. In this study, all specimens were collected at the moment of initial diagnosis (with still detectable viral RNA), which may explain the relatively low IgG and AI levels. Although IgM and IgA AI can be determined as well, these are considered less sensitive [8,17].

To our knowledge, there is currently no commercially available EV-D68 specific ELISA. The commercial ELISA that we used contains recombinant antigens from conserved and subtype-specific epitopes of the VP1 of CV-B1, CV-B3 and CV-B5 and E-6 and E-9. According to the manufacturer's information, these epitopes cross-react with other EVs, including EV-D68. However, these VP1-specific epitopes might not be the most immunodominant epitope of EV-D68, resulting in lower detection rates compared to detection of virus neutralizing antibodies. In this study, we have used the assay cut-off recommended by the manufacturer to determine positivity of antibody detection.

Altogether, we show that a quantitative EV ELISA in combination with Reiber diagram analysis and EV AI calculation can be used to detect virus-specific intrathecal antibodies in patients with EV-D68-associated CNS disease. To improve the sensitivity of detection, an EV-D68-specific ELISA with minimal cross-reactivity against other EVs should be developed. Diagnostic laboratories should consider implementing CSF EV serology to support the increasing demand of tools to identify CNS complications caused by non-polio EVs [12].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2022.105190.

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