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ORIGINAL INVESTIGATION

Hand, foot and mouth disease: seroprevalence of Coxsackie A16 and Enterovirus 71 in Germany

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Abstract Coxsackie A16 (CA16) and Enterovirus 71 (EV71) are members of the picornaviridae family and are associated with hand, foot and mouth disease (HFMD), in rare cases also to acute neurological diseases. HFMD outbreaks have been reported from many parts of the world, especially Southeast Asia. The objective of the study was to analyze CA16 and EV71 seroepidemiologically in the population of Frankfurt/M., Germany. A total of 696 individuals (349 males and 347 females, divided into seven different age groups, 1–4, 5–9, 10–14, 15–19, 20–39, 40–59 and >60 years) were tested for serum antibodies against CA16 and EV71 by the use of a microneutralization test. Sera were collected at the Frankfurt university hospital from patients suffering from other diseases between March and September 2006. CA16 and EV71 infections were observed to be widely present in the population. The age-adjusted seroprevalence for individuals ≥1 year was found to be 62.9% for CA16 and 42.8% for EV71 without a gender-specific significant difference. Only 12.0 and 27.0% of the children aged 1–4 had antibodies to EV71 and CA16, respectively – indicating that 88 and 73% of the children in this age group were susceptible to the infection. A total of 213 individuals (30.6%) was seropositive for both viruses, 303 (43.5%) showed neutralizing antibodies (NtAb) to at least one of the two viruses. A total of 180 individuals (25.9%) revealed no antibodies. High CA16 and EV71 antibody titers were found especially in the age group of the 10- to 14-year-olds, without gender-specific difference. The seroprevalence study demonstrates a common spread of CA16 and EV71 in Germany, but a relatively high susceptibility of the younger population to CA16 and EV71. Obviously, the manifestation rate, i.e., distinct disease of these infections is low.

Keywords Hand, foot and mouth disease · Seroprevalence · Coxsackie A16 · Enterovirus 71 · Neutralization assay

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Introduction

Coxsackievirus A 16 (CA16) and Enterovirus 71 (EV71) are members of the picornaviridae family. On the basis of molecular typing, EV71 can be divided into three genogroups (A, B, C), and further sub-divided into genotypes B1–5 and C1–5 [1, 2].

CA16 and EV71 are transmitted through the fecal–oral route and smear contact to throat discharges or fluid from blisters [3]. While they are commonly associated with hand, foot and mouth disease (HFMD) – sometimes only with mouth disease (herpangina) – the infections may also result in aseptic meningitis, encephalitis, myocarditis, or poliomyelitis-like paralysis. Furthermore, pulmonary edema or hemorrhage is described as complications of infection in some cases. Since the first case of EV71 infection emerged in California 1969 [4], outbreaks have followed in many parts of the world, especially in Southeast Asia. Also, in Europe, smaller outbreaks have been described [5, 6]. Up to date, a mild disease is the predominant clinical feature of infection; serious central nervous system complications are uncommon. Nevertheless, neurological involvement (e.g., encephalitis) occurs and is the most serious complication, partly with fatal outcome [7, 8].

Some fatal cases were reported for recent EV71 epidemics in Taiwan, Mongolia and China [9]. In the Mongolian outbreak 2008, 83% of the reported HFMD cases were seen in children who were younger than 10 years. Of all infected people, 10% were younger than 1 year (http://www.isid.org/ 2008).

In Germany, HFMD is a sporadic disease in children – mostly associated with CA16 infections and less common with EV71. However, epidemic waves of single enterovirus types occurred when herd immunity decreased [10]. Before eradication of poliovirus from Europe, many serum surveys were performed to assure immunity [11–14]; only few of them covered non-polio enteroviruses [3, 11]. A serum survey on CA16 is still missing. In Europe, non-polio enteroviruses remain an important cause of illness in the absence of vaccine and effective antiviral therapy [15–17].

Therefore, this study was performed to get additional information on the seroprevalence of enteroviruses CA16 and EV71 in Germany, i.e., to rule out population's immunity and to allow a direct comparison of CA16 and EV71 seroprevalence in the same cohort.

Methods

Collective

A representative panel of 696 serum samples were obtained from patients who had been serologically tested for other viral infections or immune status at the Institute of Medical Virology, Johann Wolfgang Goethe University in Frankfurt/Main. Sera from Germans as well as from foreigners, who lived in Germany, were included in the survey. Inclusion criteria were age and sex. For age distribution, seven age groups were chosen (1–4, 5–9, 10–14, 15–19, 20–39, 40–59 and >60 years), with each group having 48–50 females and 49–50 males. The sera were collected between March and September 2006 and stored at −20°C until testing. Exclusion criteria were suspected or laboratory-proved enterovirus infection.

Neutralization assay

A microneutralization test using CA16 and EV71 was performed as previously described [18, 19]. Serum of known CA16 and EV71 neutralizing activity was included in each test to examine reproducibility of results. Briefly, sera were inactivated at 56°C for 30 min before use, diluted twofold from 1:10 to 1:1,280, and then incubated for 1 h at 37°C (in a CO_2 incubator) with 100 tissue culture infective $dose_{50}$ ($TCID_{50}$) of challenge virus (either CA16 or EV71). CA16 and EV71 (genogroup C2) virus isolates (*kindly provided by Dr. Sabine Diedrich, German reference centre for poliomyelitis and enteroviruses", Robert Koch Institute, Berlin*) had been characterized previously by specific monoclonal antibodies using immunofluorescence technique as well as by cDNA sequence analysis in the VP1 gene region subsequent to specific nucleic acid amplification by RT–PCR [3]. After the incubation period, 50 µl of the serum-virus suspension was added to monolayer of 1 day old human epithelial colorectal adenocarcinoma cells ($CaCo_2$). Cell controls and a reference serum sample were included in each batch. Each test serum was investigated in duplicate. After incubation for 10 days, the highest dilution of serum that prevented the development of cytopathic effects (CPE) in both wells was recorded. A serum sample was considered positive, if antibodies were present at a dilution 1:≥10 of the inoculated serum specimen. If the final serum dilution of the duplicates differs more than one stage, the result was rejected and the test was repeated.

Calculation of the antibody prevalence

For statistical analysis, 95% confidence intervals [CI] for proportions and the chi-square test (Yates rectified) were calculated. Differences with an error probability of $P < 0.05$ were regarded as significant. The statistical analysis was performed using the BiAS program for Windows 8.3 (Epsilon Verlag, Hochheim Darmstadt 2007).

Results

Overall seroprevalence of CA16 and EV71

CA16 and EV71 infections are widely present in the population: The age-adjusted CA16 seroprevalence for individuals ≥1 year was found to be 62.9% (CI 59.2–66.5%) for CA16 and 42.8% (CI 39.1–46.6%) for EV71. The CA16 seroprevalence was significantly higher than that for EV71 in individuals ≥1 year old ($P < 0.001$).

No significant gender-specific difference in seroprevalence was observed for both CA16 and EV71 – 62.8% (218/347) of the females and 63.0% (220/349) of the males ($P = 0.98$) showed neutralizing antibodies to CA16, while 44.4% (154/347 females) and 41.3% (144/349 males) ($P = 0.45$) were tested EV71 seropositive (Fig. 1).

Age-dependent seroprevalence of CA16 and EV71

The analysis of the different age groups ($n = 98$–100) revealed that CA16 seroprevalence increased from 27.0% in the first age group (1–4 years) to 52.0% in the subsequent group (5–9 years old), and thereafter to 83.0% in the 40–59-year-old individuals. In the age group of the 10–14-year-old individuals, a plateau (73.5% seropositive) relative

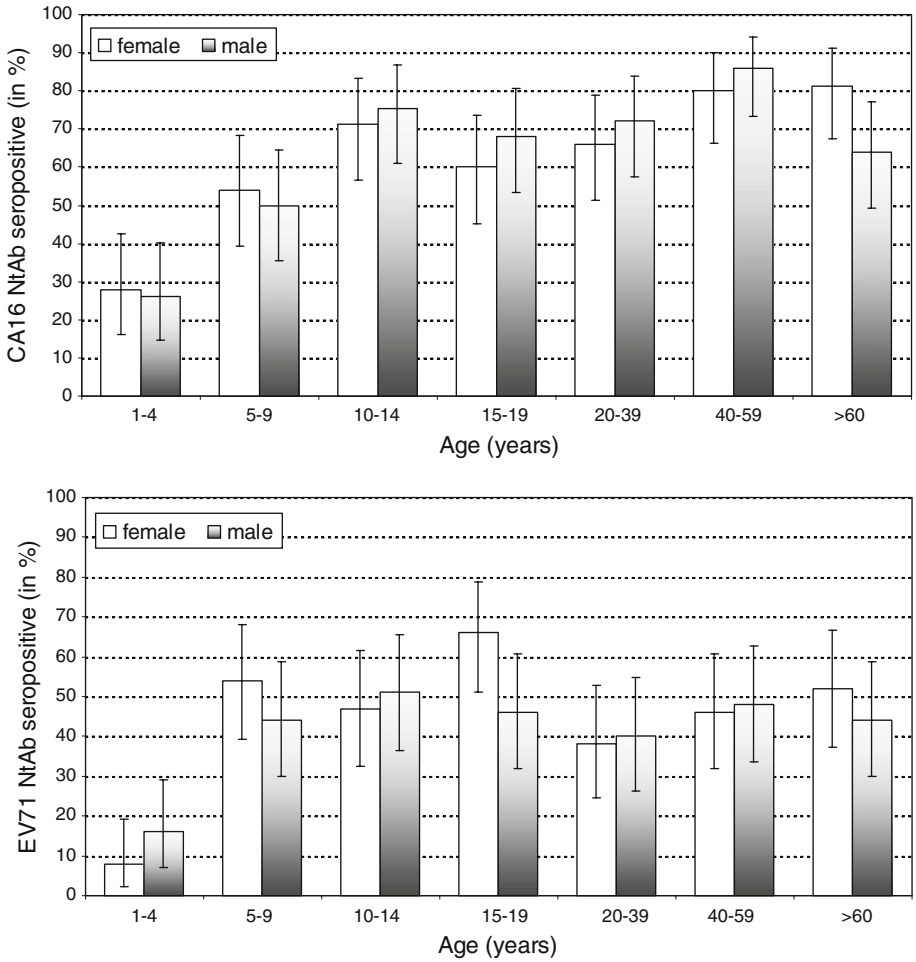

Fig. 1 Age-related and sex-dependent prevalence of neutralizing serum antibodies to CA16 (*upper panel*) and EV71 (*lower panel*) in individuals living in Frankfurt, Germany (female – *white bars*; male – *grey bars*; the *lines* indicate 95% confidence intervals)

to that attained by individuals in the other age groups [ranging from 15–19-year-old (64.0%) to the >60-year-old individuals (72.4%)] was reached (Fig. 1 – upper panel). There was no significant gender-specific difference.

In comparison to CA16, the overall seroprevalence of EV71 was found lower in most age groups. There was a significant increase in the EV71 seroprevalence from 12.0% among 1- to 4-year-olds to 49.0% among the 5- to 9-year-olds ($P < 0.001$). The EV71 seroprevalence attained its peak in this age group (5–9-years) with no further significant rise or decline in the subsequent age groups (Fig. 1–lower panel). However, a little, but no significant gender-specific difference in seroprevalence was observed in the age group 15–19 years (female > male) as indicated by the 95% CIs.

Age-dependent immunity to HFMD

A total of 30.6% (CI 27.2–34.2%; 112/347 females and 101/349 males; $P = 0.38$) of all tested individuals revealed neutralizing antibodies against both virus types, while 43.5% (CI 39.8–47.3%; 141/347 females and 162/349 males; $P = 0.14$) were seropositive only for one of both viruses. Of all tested individuals, 180 (25.9%) (CI: 22.6–29.3%; 94/347 females and 86/349 males; $P = 0.52$) had no detectable antibodies against CA16 or EV71 (Fig. 2). There was an age-related decrease of CA16/EV71 seronegative individuals. Conversely, the fraction of individuals seropositive for one or both of the tested viruses increased.

Titer distribution of neutralizing antibodies in seropositive individuals

To analyze the immunity level, three NtAb titer ranges were defined: 1:10–1:20 (low level), 1:40–1:320 (medium level) and 1:640 to 1:≥1,280 (high level).

The analysis of the titers of NtAb against CA16 and EV71 independent of age and gender showed that all CA16 NtAb levels were approximately equally distributed. In contrast, patients presenting low-level EV71 NtAb dominated while high-level EV71 NtAb levels were significantly less likely (Fig. 3).

Considering the percentage distribution of NtAb levels of all NtAb positive patients, a significant difference in the distribution pattern between CA16 and EV71 (Fig. 3 – right panel) was observed. From the 438 CA16 NtAb positive

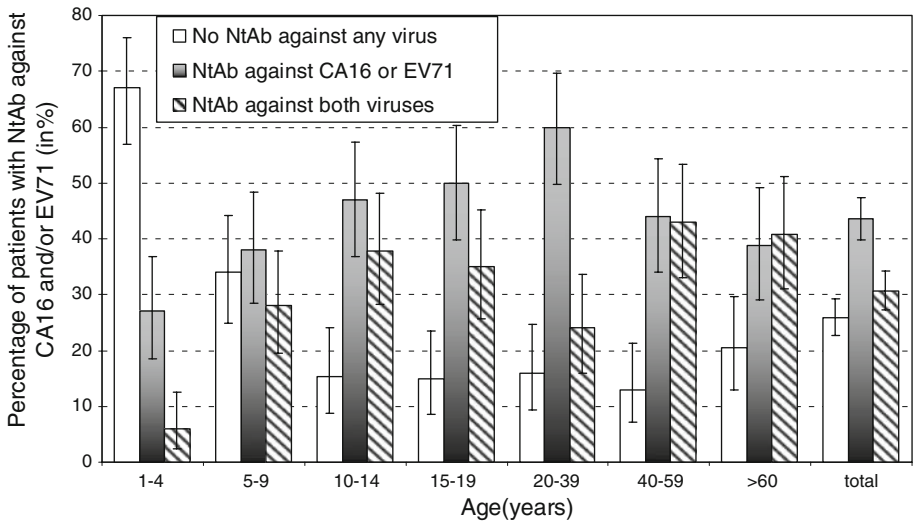

Fig. 2 Age-specific fractions of individuals revealing complete (CA16 + EV71), partial (either CA16 or EV71) or no immunity to HFMD (neither CA16 nor EV71). The *lines* indicate 95% confidence intervals

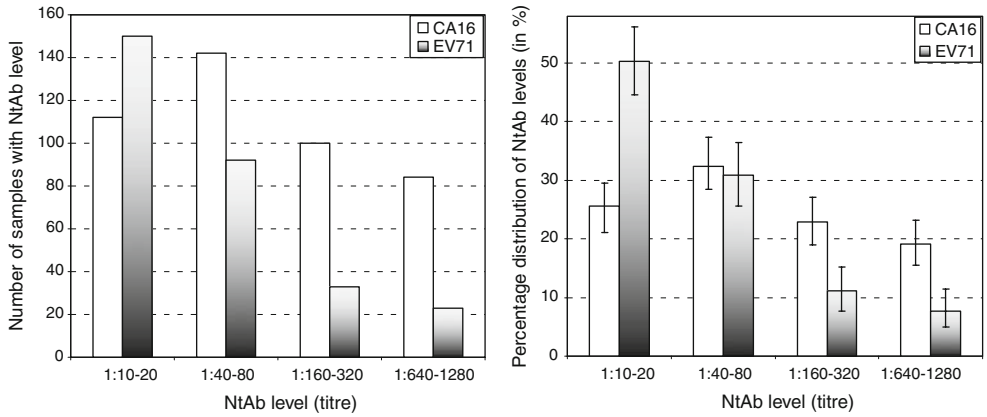

Fig. 3 Titer distribution of seropositive individuals. *Left panel* number of individuals with a specific titer level, *right panel* percentage of individuals with a specific titer level (the *lines* indicate 95% confidence intervals)

sera, 19.0% presented high level NtAb (1:640–1:1,280), 55% medium level (1:40–1:320) and 26% low level (1:10–1:20), while for the 298 EV71 seropositives, 8.0% had high level, 42% medium level and 50% low level NtAb.

Age-dependent level of immunity to CA16 and EV71 infections

The analysis of the immunity level in relation to the age of the individuals showed that the number of sera with high NtAb levels to CA16 was highest in the age group of 10- to 14-year-olds followed by the 40- to 59-year-olds. The number of individuals with low NtAb level increased with age – except the 40–59-year-old group (Fig. 4 – upper panel).

In contrast to CA16, EV71 seropositive individuals generally showed lower NtAb titers. With increasing age, the number of individuals presenting medium-level NtAb titers decreased. High EV71 antibody level was mostly present in the group of 10- to 14-year-olds (Fig. 4 – lower panel). There were no significant gender-specific differences (data not shown).

Discussion

CA16 and EV71 are associated with sporadic cases and epidemics of HFMD. Between 1970 and 2000, three separate waves of EV71 activity emerged in the world, one in each decade [20]. The most severe EV71 epidemic occurred in Taiwan in 1998 – more than 130,000 cases of HFMD and fatal EV71 cases were reported [21, 22]. In China, a still ongoing HFMD epidemic has affected more than 30,000 children [23]. Other parts of Asia (e.g., Mongolia, Vietnam) were also involved, mainly affecting children [2, 9]. Many thousands of infected people were reported in 2007–2008 (http://www.isid.org/ 2008). Furthermore, sporadic EV71 cases have been reported from several European countries [5, 6, 24–27]. In some of these EV71 cases, genotype C4 was identified. This genotype was reportedly associated

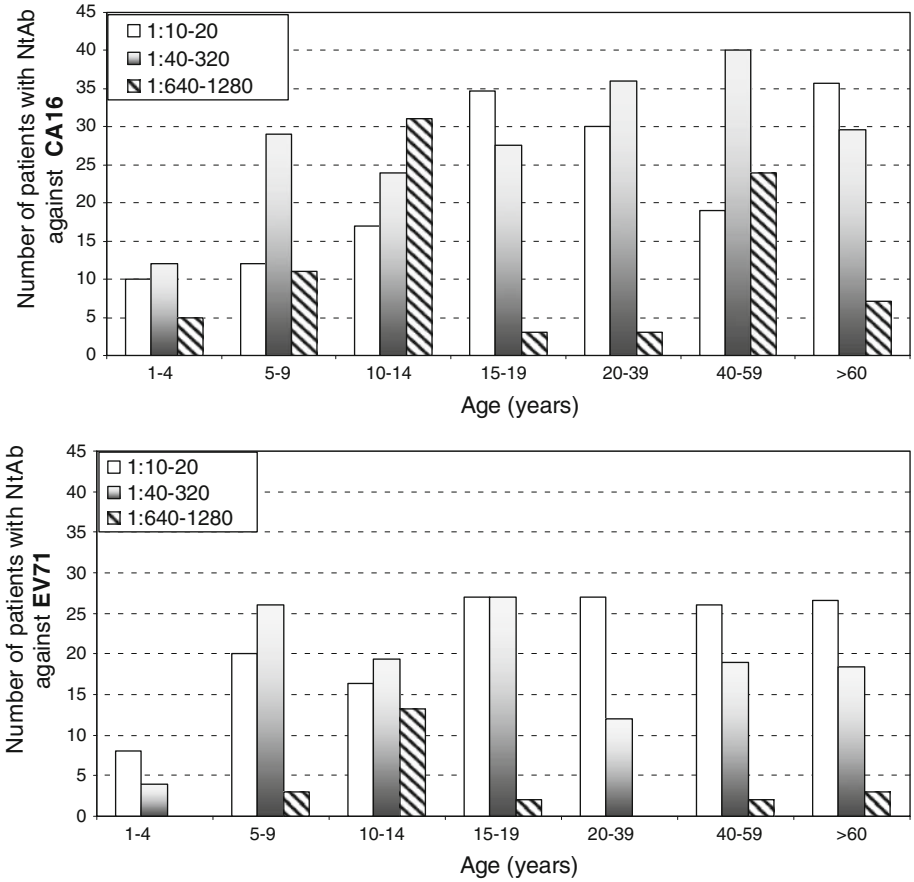

Fig. 4 Titers of NtAb to CA16 (*upper panel*) and EV71 (*lower panel*) in relation to age. Only seropositive individuals of the study are included in the figure

with the recent high mortality in China [5]. EV71 was also the most important virus responsible for outbreaks of acute neurological diseases in Australia [28–31] and the United States [32, 33]. Even though no genotype-specific sequences which are responsible for neurovirulence could be identified, the strains causing brain stem encephalitis and pulmonary edema in the Far East are similar and have arisen since 1997 [34]. Different EV71 genotype analyses had shown that EV71 genotypes have changed with time in the United States and Japan [35] and are co-circulating worldwide. In Germany, genogroup B circulated before 2000, followed by strains which predominantly belonged to the C1 (in the years 2000–2005) and C2 genogroup (in the years 2006–2007). Overall, genogroup C1 is predominating in Europe [3]. In Germany as well as in the United States, E71 belongs to the 15 most common enterovirus serotypes [3, 36].

CA16 co-circulated with EV71, however, without causing any severe illnesses [34]. EV71 illness seems to be more severe with significantly greater frequency of serious complications and fatality than illness caused by CA16 [37]. Thus, CA16 and EV71 infection have emerged as an important public problem. While in temperate climates, enteroviruses' outbreaks mostly take place during summer and autumn, in tropical areas, infections occur with high incidence throughout the year [38, 39].

Our investigations revealed an endemic spread of CA16 and EV71 infections in Germany similar to other parts of the world [34, 40, 41]. The EV71 NtAb assay used for this study detects specific antibodies regardless of the genogroup (antigenic cross-reactivity) [42].

The age-adjusted seroprevalence of CA16 significantly exceeds that of EV71 (62.9 vs. 42.8%). Epidemic waves could not be traced by this cross-sectional study. Neither for CA16 nor for EV71 could a gender-specific significant difference be seen (CA16–62.8% ♀ vs. 63.0% ♂; EV71–44.4% ♀ vs. 41.3% ♂). Our data show that 88% of the children aged 1–4 are susceptible to EV71 while about 2/3 of all 1- to 4-year-olds are susceptible to CA16 (Fig. 1). There are close similarities between CA16 and Coxsackie A9 on the one hand and EV71 and Coxsackie B3 on the other hand when our data are compared with age-related prevalence rates of other enteroviruses in Germany [11]. Our data also show that most of the infections are acquired during childhood (CA16) or early adolescence (EV71). In these age groups, the highest antibody titers were observed. The slope of CA16 and EV71 seroprevalence in young age groups indicates that infection beyond (pre)school years is uncommon. This finding is supported by the observation that a high proportion of children <5 years old were seronegative. The positive immune status of children reached a

steady state in the age groups 5–9-years and 10–14-years for EV71 and CA16, respectively. Furthermore, the number of individuals with high level anti-EV71 NtAb declined with age. This means that reinfection of the elderly is rare; otherwise, the number of individuals with high level NtAb should increase with age. In contrast to EV71, a second peak of high NtAb level was recorded for CA16 in the 40- to 50-year-old group, indicating a second, probably smaller epidemic wave; the overall seroprevalence curve of CA16 was not affected, as mentioned earlier. Seroprevalences of enteroviruses are surprisingly stable over decades, as previously shown for coxsackievirus B types [11, 18].

Immunity to CA16 and EV71 is mainly dependent on humoral factors, i.e., formation of neutralizing serum antibodies, although it is known that enterovirus infections also induce T-cell immunity [43]. It is not clear at present whether every individual with a low level immunity or no detectable neutralizing antibody is susceptible to infection. Particularly, the elderly might be protected by a long-lasting immunity even if antibody titer has declined – otherwise, persistent high antibody titers would be detectable throughout life.

Concerning immune protection against HFMD, we found that 30.6% of all tested individuals had antibodies both to CA16 and to EV71, while 43.5% were reactive only against one of both viruses.

While CA16 seroprevalence in Germany is recorded by us for the first time, EV71 seroprevalence was recently investigated by another German group [3]. They found among 6- to 10-year-old children 56.4%, among children aged 10–15 67.2% seropositive. Comparable age groups in our collective showed a prevalence of 49% each. Taking the 95% CI into account, there is no significant difference between both studies. In the report of Diedrich et al. [3], a maximum rate of seropositivity (75%) was recorded in individuals aged 20–40. The level remained stable in elderly groups. In our study, positive immune status peaked in a younger age group (5–9 years old) on a lower level (49%; 95% CI 38.9–59.2%). There might be some reasons for this discrepancy: In the study of Diedrich et al. [3], "randomly selected stored serum samples from healthy children and adults who participated at a survey in 1997/1998" were used, while our samples were collected in 2006. So, a time-of-sample-related change in seroprevalence in Germany cannot be excluded.

In conclusion, CA16 and EV71 infections are common in children and are acquired largely in the (pre)school years. Spread of these viruses is lower in the other age groups. The seroprevalence data show a continuous circulation of CA16 and EV71 in Germany similar to the majority of other enteroviruses [11]. Although EV71 is not often isolated in Europe and the seroprevalence is lower than for CA16, it is, however, an important enterovirus for differential diagnosis of neurological diseases such as meningitis and paralytic disease.

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