Neutralizing Antibody against Enterovirus D68 in Children and Adults before 2014 Outbreak, Kansas City, Missouri, USA[1]

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We evaluated enterovirus D68 seroprevalence in Kansas City, Missouri, USA, from samples obtained during 2012–2013. Neutralizing antibodies against Fermon and the dominant 2014 Missouri isolate were universally detected. Titers increased with age. Widespread circulation of enterovirus D68 occurred before the 2014 outbreak. Research is needed to determine a surrogate of protection.

The first enterovirus D68 (EV-D68) isolate (Fermon) was identified in 1962 (*1*,*2*). Before a 2014 EV-D68 outbreak, US reports of EV-D68 were relatively sparse (<100 sporadic cases and periodic outbreaks in 50 years) (*3*). In autumn 2014, a total of 1,153 confirmed EV-D68 cases occurred in 49 states and the District of Columbia; EV-D68 patients mostly had respiratory symptoms consistent with those in previous EV-D68 outbreaks and cases (*4*). Nationwide, severe disease occurred mostly in school-aged children. Through September 2014, EV-D68 was detected in 338 of 551 children with rhinovirus/enterovirus-positive test results; most (61.3%) were hospitalized at The Children's Mercy Hospital (Kansas City, MO, USA). Hospitalized EV-D68 patients often had asthma or recurrent wheezing. Many of these EV-D68–infected children had unusually severe, refractory bronchospasms, which resulted in 100 intensive care unit stays (*5*).

The Study

We assessed the prevalence of EV-D68 neutralizing antibody in the Kansas City population before the 2014 outbreak using deidentified banked serum samples (stored at The Children's Mercy Hospital) collected in 2012 (n = 155) and 2013 (n = 281) from healthy persons >2 years of age during a poliovirus seroprevalence study (*6*). Age, sex, and race/ethnicity distributions matched 2010 Kansas City census data (Appendix, https://wwwnc.cdc.gov/EID/article/25/3/18-0960-App1.pdf) (*6*).

We performed serology testing at the Centers for Disease Control and Prevention (Atlanta, Georgia, USA). We used an adapted poliovirus microneutralization assay to test samples for neutralizing antibodies (*6*,*7*) against 4 phylogenetically distinct EV-D68 isolates: Fermon (GenBank accession no. NC038308); the 2014 Missouri isolate 14-18949 (clade B1, GenBank accession no. KM851227); and 2 related but non-Missouri 2014 isolates, 14-18952 (clade B2) and 14-18953 (clade A2; GenBank accession nos. KM851230–1; Figure 1; Appendix) (*9*). The proportion of US patients from whom these three 2014 circulating strains were detected was >91% for 14-18949, 7.4% for 14-18952, and <2% for 14-18953 (*10*).

Besides age, sex, and race/ethnicity (Hispanic vs. non-Hispanic), population demographics are descriptive; small subset numbers precluded formal statistical analysis. We performed analyses using Sigmaplot version 12.2 (http://www.sigmaplot.co.uk/index.php; Appendix), and we considered p values <0.05 significant.

Of 436 serum samples, 217 were from male donors; median age was 13 (range 2–81) years. All had neutralizing antibody (i.e., $\geq 3 \log_2$, ≥1:8 titer) against Fermon and 14-18949 (Table); 97% of samples had neutralizing antibody to 14-18953 and 89% to 14-18952. Overall seropositivity for the 4 isolates was not different (p = 0.763).

In total, 50% (24/48) of the 14-18952–seronegative samples and 57% (24/42) of the 14-18953–seronegative samples (p<0.001) came from male donors. Our 2–5-year-old age group made up the largest proportions of these seronegative populations (67% [32/48] of 14-18952 and 36% [15/42] of 14-18953; p = 0.003). The 21 persons seronegative for both 14–18952 and 14–18953 had a median age of 3 (2–61) years. Donor sex and race/ethnicity and season of sample acquisition did not differ between samples seropositive and seronegative for 14-18952 or 14-18953 (data not shown).

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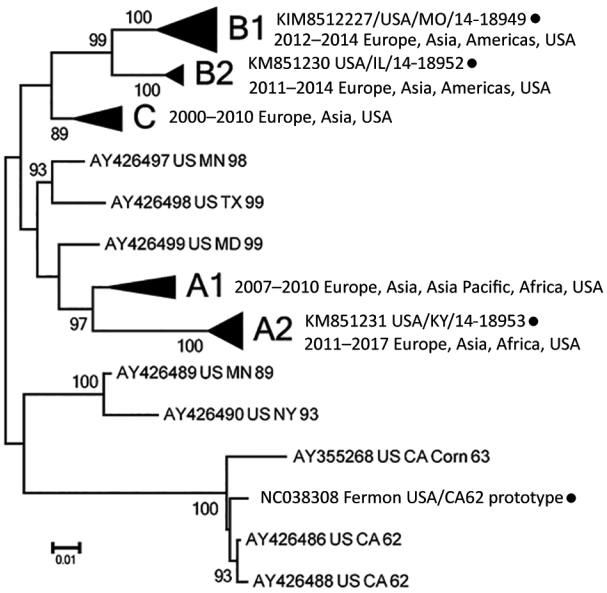

Figure 1. Unrooted neighbor-joining tree depicting phylogenetic relatedness of reference enterovirus D68 isolates (black circles) used in microneutralization assays in study of enterovirus D68 seroprevalence among children and adults in Kansas City, Missouri, USA, 2012–2013. We constructed tree using complete virus protein 1 gene sequences and MEGA6.0 (*8*). Branching within major clades (bolded) is collapsed for clarity.

Neutralizing antibody titers also did not differ by season of sample acquisition, donor race/ethnicity, or donor sex. Mean titers to 14-18949 were lower ($p<0.05$) among self-identifying Hispanics (n = 36; 7.1 $\log_2$, range 3.83–10.5 $\log_2$) than among non-Hispanics (n = 400; 7.83 $\log_2$, range 3.17–10.5 $\log_2$), but this difference might not be clinically significant. Median titers rose with each advancing age group, except against Fermon among 11–15-year-olds ($p<0.001$; Table). The overall median titer was highest for 14-18952 (8.34 $\log_2$, range 2.5–10.5 $\log_2$; $p<0.001$), despite a comparatively lower seropositivity (89%). The overall titer was lowest for 14-18953 (6.83 $\log_2$, range 2.83–10.5 $\log_2$; $p<0.001$), despite a relatively high seropositivity (91%).

All serum samples had neutralizing antibody against the major EV-D68 isolates circulating in the United States in 2014, and most had antibody to the other 2 less frequently detected isolates that year (Figure 2; Appendix Figure). During the 2014 outbreak, 5–10-year-olds (who would have been 3–8-year-olds during the sampling time of our study) had the most severe disease. Severe EV-D68 disease occurred often in children with atopic disease, reactive airway disease, or asthma. In 2014, little EV-D68 disease was noted among adolescents, adults, or the elderly (*4*). The age-associated severe EV-D68 respiratory disease observed in 2014 parallels our finding of lower overall titers in 2–5-year-olds and 6–10-year-olds.

Although introduction of EV-D68 into naive populations could have explained the 2014 outbreak, universal detection of antibody against 14-18949 (dominant 2014 isolate) before 2014 indicates previous widespread exposure to 14-18949 or a related isolate. EV-D68 was also detected in Kansas City as early as 2009 (F. Hassan, University of Missouri at Kansas City, pers. comm., May 2018). Thus, the Kansas City 2014 outbreak did not occur because of population naivete to a 14-18949–like isolate. Indeed, neutralizing antibodies to other EV-D68 isolates were also detected (*4*,*10*). That 2–5-year-olds in our study had lower titers to 14-18949 (Figure 2) suggests that older persons had more experience with 14-18949 or confirms that antibody elicited by non–14-18949 isolates can also neutralize 14-18949. Severe disease during the 2014 outbreak occurred among children who, according to our results, were relatively experienced with this pathogen; they were positive for neutralizing antibodies against 14-18949 but had lower median titers and a reduced reverse cumulative distribution compared with other age groups.

Why this large outbreak was able to occur in a population with a high prevalence of neutralizing antibody against the outbreak isolates remains unclear. One possibility is that respiratory tract mucosal antibody (probably in the form of secretory IgA) is more relevant than serum antibody for protection against respiratory disease (*11*). In this study, we could not address this possibility because only serum samples were available for testing. Also, certain persons could be more susceptible to severe disease because of genetic factors, preexisting atopy or asthma, or differences in other parts of the immune response, including immunopathologic responses. The argument for multiple factors contributing to disease despite the presence of neutralizing antibody is bolstered by the predilection of persons with asthma or atopic disease to have severe disease (*5*); further, asthma patients experience more tight junction injury than persons without asthma during rhinovirus infection (*12*).

The only demographic factor potentially affecting titers was Hispanic race/ethnicity. However, the ≈0.8 $\log_2$ difference might not be clinically significant, considering the median titers in both groups were >5 $\log_2$; thus, whether race/ethnicity is a factor is unclear.

Our data parallel another study with a similar 2011 sampling timeframe conducted in China (*13*). In that study, neutralizing titers against Beijing/2008/01 EV-D68 were low in serum samples collected in 2004 for all age groups, but their 2011 titers resembled our data, despite few reported EV-D68 illnesses in the sampled area during 2007–2011. Their lowest overall titers were also observed in persons of younger age groups.

EV-D68 has been proposed as a cause of acute flaccid myelitis. Increasing reports of this condition underscore the need to better understand EV-D68 seroprevalence and circulation (*14*).

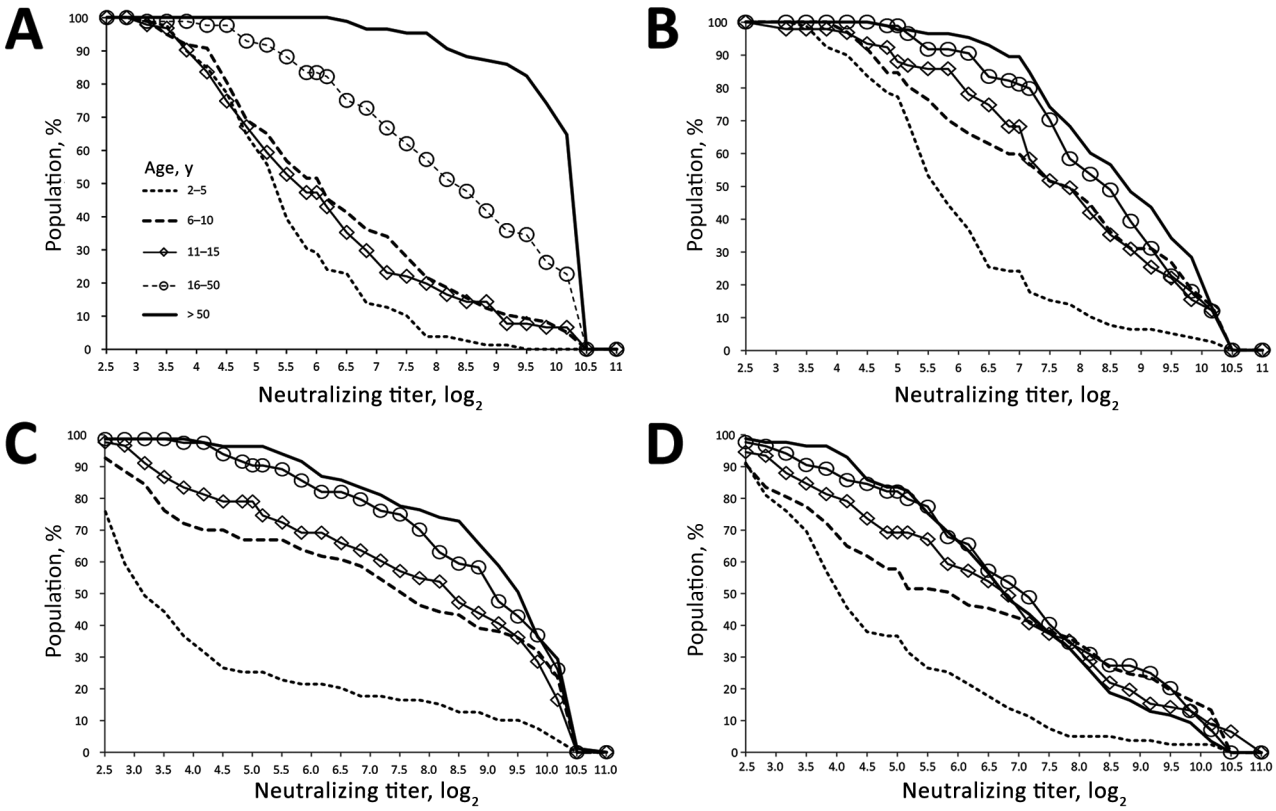

Figure 2. Reverse cumulative distribution curves of neutralizing antibody titers against enterovirus D68 isolates, by isolate and age group, Kansas City, Missouri, USA, 2012–2013. A) Fermon; B) dominant 2014 isolate 14-18949; C) less frequently circulating 2014 isolate 14-18952; D) rare 2014 isolate 14-18953. The reverse cumulative distribution pattern for 14-18949 varied the least by age group.

Table. Neutralizing antibody positivity and titers for each enterovirus D68 isolate, by age group, Kansas City, Missouri, 2012–2013

		% Neutralizing antibody positive; median (range) neutralizing antibody titer*			
Age group, y	No.	Fermon	14-18949	14-18952	14-18953
2–5	79	100; 5.50 (3.17–9.5)	100; 5.83 (3.5–10.5)	60; 3.17 (2.5–10.5)	81; 4.17 (2.5–10.5)
6–10	97	100; 6.17 (3.17–10.5)	100; 7.83 (4.17–10.5)	89; 7.83 (2.5–10.5)	83; 6.17 (2.5–10.5)
11–15	91	100; 5.83 (3.17–10.5)	100; 7.83 (3.17–10.5)	97; 8.50 (2.5–10.5)	93; 6.50 (2.5–10.5)
16–50	84	100; 8.50 (3.83–10.5)	100; 8.50 (4.83–10.5)	98; 9.17 (2.5–10.5)	96; 7.17 (2.5–10.5)
>50	85	100; 10.50 (6.5–10.5)	100; 8.83 (4.83–10.5)	99; 9.50 (2.5–10.5)	98; 6.83 (2.5–10.5)
Total	436	100; 6.83 (2.83–10.5)	100; 7.83 (3.5–10.5)	89; 8.34 (2.5–10.5)	91; 6.50 (2.5–10.5)

*Antibody titers were measured by using the cell viability kit ATPlite (Perkin Elmer, http://www.perkinelmer.com); the titers shown are the $\log_2$ inverse dilution of the lowest antibody concentration with luminescent activity.

Our study had several limitations (Appendix). Because of the retrospective study design, our data and interpretations are limited regionally and temporally. We tested for only 4 select EV-D68 isolates, and antibody reactivity with other isolates might differ.

Conclusions

We detected at least some neutralizing antibody to Fermon and the dominant 2014 isolate (14-18949) in all 436 EV-D68 samples acquired during 2012–2013 in Kansas City. Prospective studies are warranted to define a protective threshold of serum neutralizing antibody (or a surrogate of protection), the distribution of titers in children <2 years of age, and whether antibody levels differ by race/ethnicity.

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Neutralizing Antibody against Enterovirus D68 in Children and Adults before 2014 Outbreak, Kansas City, Missouri, USA

Appendix

Microneutralization Assay

The microneutralizion assay was adapted from an established poliovirus antibody assay (*1*,*2*). In brief, 2-fold serum dilutions, 1:8 to 1:1,024, were combined with 100 cell culture ID_{50} of enterovirus D68 (EV-D68) to enable antibody to bind to virus. After a 3-hour incubation, each virus–serum mixture was inoculated onto rhabdomyosarcoma (CCL-136; American Type Culture Collection, https://www.atcc.org) cell monolayers. Each serum dilution was tested in triplicate against each isolate. Each run had a known positive control serum (horse anti-Fermon antibody), and multiple (at least 4) positive control replicates were distributed across each run. When >7 serum samples were tested in the same run, sample position was randomized via a balanced block randomization scheme. Each run included 2 control plates with no serum or control antibody; rhabdomyosarcoma cells alone served as a no-virus control. A back-titration virus–control plate was used for each of the 4 EV-D68 isolates to confirm the amount of antigen used in each run. A luminescent cell viability kit (ATPlite; Perkin Elmer, http://www.perkinelmer.com) was used to evaluate neutralization. Samples with luminescent activity at a titer of greater than 3 $\log_2$ (1:8 dilution) were considered positive for neutralizing antibody.

Demographics and Data Analysis

We used the following age groups: 2–5-year-olds (n = 79), 6–10-year-olds (n = 97), 11–15-year-olds (n = 91), 16–50-year-olds (n = 84), and 51–81-year-olds (n = 85). Race/ethnicity was self-reported, and for analysis, we used the groups white (n = 330), black (n = 66), mixed race (n = 19), other (n = 10), Asian (n = 7), Native American (n = 5), and Pacific Islander (n =

1). In total, 36 participants were Hispanic/Latino and 396 were non-Hispanic/Latino; 10 declined to report race, and 6 declined to give ethnicity.

For analysis, the population was age stratified. The age and sex distribution and season of sample acquisition were not different between Hispanics and non-Hispanics. Categorical values were analyzed by the χ^2 test. Overall antibody titers were analyzed by the Kruskal-Wallis rank-sum test to determine if values significantly differed between groups, with subset comparisons performed by using the Kolmogorov-Smirnov test. Differences between isolates were assessed by using nonparametric analysis of variance, and adjustments for multiple comparisons was performed by using Tukey-Kramer comparisons.

Reverse Cumulative Distribution

For the Fermon isolate, the titers of the >50-year-old age group were significantly higher than those of the 4 younger age groups ($p<0.001$ for all; Figure 1, panel A, main text). Likewise, the titers of the 16–50-year-old age group were higher than those of the 3 younger age groups ($p<0.001$ for all 3). The titers to 14–18949 among >50-year-olds were similar to those of the 16–50-year-olds but higher than those of the 3 youngest age groups ($p<0.001$ for all; Figure 1, panel B, main text). The 14–18949 titers among 2–5-year-olds were significantly lower than those of all other age groups. Titers to 14–18952 (Figure 1, panel C, main text) and 14–18953 (Figure 1, panel D, main text) were significantly lower in the 2–5-year-old age group than the 4 older age groups ($p<0.001$ for all). Titers to 14–18952 were also lower in the 6–10-year-old age group than the 3 oldest age groups ($p<0.001$ for all 3).

Comparisons of isolate seropositivity by age group (Appendix Figure, panels A–E) revealed that 2–5-year-olds had higher titers for both Fermon and 14–18949 than 14–18952 and 14–18953 ($p<0.001$ for all; Appendix Figure, panel A). In the 6–10-year-old age group, titers for 14–18949 were higher than those for both Fermon and 14–18953 but not different from those for 14–18952 (Appendix Figure, panel B). In the 11–15-year-old age group, titers for 14–18949 and 14–18952 did not differ but were higher than those for both Fermon and 14–18953 ($p<0.001$ for all; Appendix Figure, panel C). In the 16–50-year-old age group, the only difference noted was that titers for 14–18953 were lower than those for the other 3 isolates ($p<0.001$ for all; Appendix Figure, panel D). In the >50-year-old age group, titers for Fermon were higher than those for the

other 3 isolates, and titers for 14–18953 were lower than those for the other 3 isolates ($p<0.001$ for all; Appendix Figure, panel E).

Limitations

Limitations of our study include the retrospective study design and the use of deidentified samples stored since 2012–2013. We were not able to determine if seroprevalence differed at an earlier time point, e.g., in 2004 as in the study conducted in China (*3*). We have no clinical data for serum sample donors, e.g., information regarding comorbidities, such as asthma and atopy, or whether they got EV-D68 illness during 2014. We tested for neutralizing antibody against only 4 EV-D68 isolates, so patterns of neutralizing activity against other EV-D68 isolates could differ. However, we did test for the isolates known to have circulated in Kansas City, Missouri, USA, in 2014. Age ranges for our groups could be considered arbitrary; the age groups we used were adapted from those originally chosen to evaluate seroprevalence of poliovirus, another enterovirus. Our age groups parallel those in the Beijing report (*3*). We had no samples from children <2 years of age, who would most likely have been EV-D68 naive. The racial, ethnic, and age distributions of our population matched those of Kansas City census data and, therefore, might not be generalizable to other geographic areas. That said, these distributions closely mirrored those of the United States as a whole in 2010.

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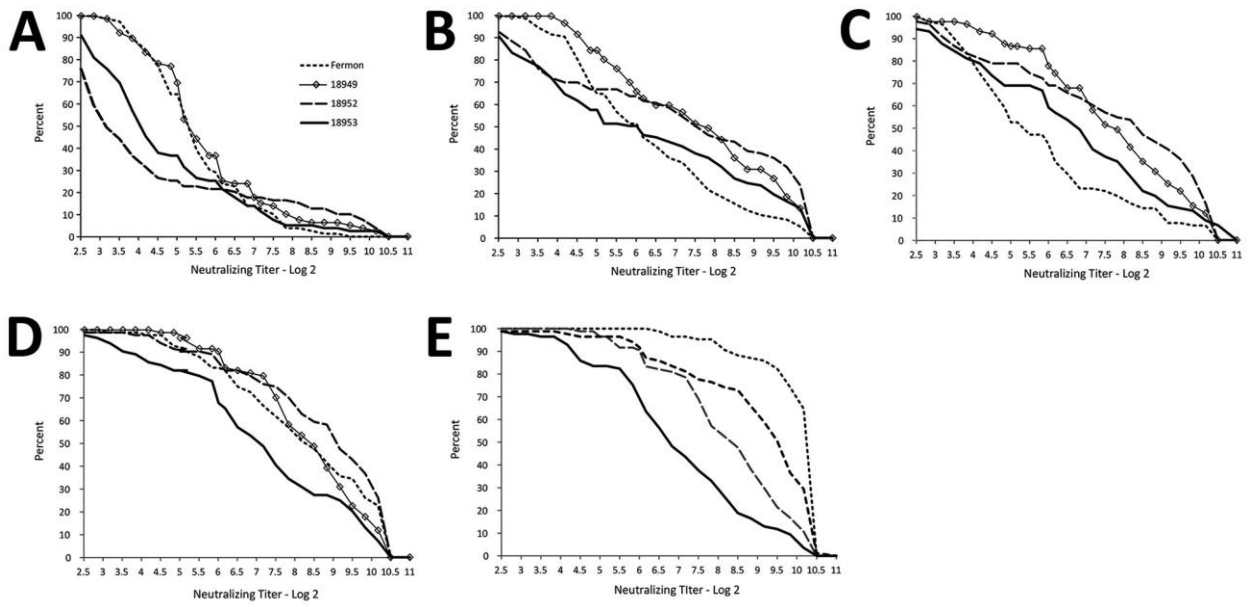

Appendix Figure. Reverse cumulative distribution (RCD) curves of enterovirus D68 (EV-D68) neutralizing antibody titers against 4 EV-D68 isolates, by age group, Kansas City, Missouri, USA, 2012–2013. A titer >3.0 $\log_2$ was considered positive for neutralizing antibody. RCDs represent the proportion of the population with a titer at least as high as the value on the *x* axis. Data from A) 2–5-year-olds; B) 6–10-year-olds; C) 11–15-year-olds; D) 15–50-year-olds; and E) >50-year-olds. Persons in the middle 3 age groups (6–50-year-olds) had the most similar RCD patterns.