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RESEARCH ARTICLE
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Development and Use of an Endpoint Titration Assay To Characterize Mumps IgG Avidity following Measles, Mumps, and Rubella Vaccination and Wild-Type Mumps Infection

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ABSTRACT Waning mumps IgG antibody and incomplete IgG avidity maturation may increase susceptibility to mumps virus infection in some vaccinees. To measure mumps IgG avidity, serum specimens serially diluted to the endpoint were incubated on a commercial mumps-specific IgG enzyme immunoassay and treated with the protein denaturant diethylamine (60 mM, pH 10). End titer avidity indices (etAIs [percent ratio of detected diethylamine-resistant IgG at endpoint]) were calculated. Unpaired serum specimens ($n = 108$) from 15-month-old children living in a low-incidence setting were collected 1 month and 2 years after the first measles, mumps, and rubella vaccine dose (MMR1) and tested for mumps avidity. Per the receiver operating characteristic curve, the avidity assay is accurate (area under the curve, 0.994; 95% confidence interval [CI], 0.956 to 1.000), 96.5% sensitive (95% CI, 87.9 to 99.6%), and 92.2% specific (95% CI, 81.1 to 97.8%) at an etAI of 30%. When 9 sets of paired serum specimens collected 1 to 60 months post-MMR1 were tested for mumps and measles IgG avidity using comparable methods, the mumps etAI increased from 11% to 40 to 60% in 6 months. From 6 to 60 months, avidity was sustained at a mean etAI of 50% (95% CI, 46 to 54%), significantly lower ($P < 0.0001$) than the mean measles etAI of 80% (95% CI, 74 to 86%). Mean etAIs in children 2 years post-MMR1 ($n = 51$), unvaccinated adults with distant mumps disease ($n = 29$), and confirmed mumps cases ($n = 23$) were 54, 62, and 57%, respectively. A mumps-specific endpoint avidity assay was developed and validated, and mumps avidity was determined to be generally sustained at etAIs of 40 to 60%, reaching etAIs of >80% in some individuals.

IMPORTANCE Numerous outbreaks of mumps have occurred in the United States among two-dose measles-mumps-rubella (MMR)-vaccinated populations since 2006. The avidity of mumps-specific IgG antibodies may affect susceptibility to mumps virus infection in some vaccinated individuals. To accurately measure mumps avidity, we developed and validated a mumps-specific IgG avidity assay that determines avidity at the endpoint titer of serially diluted serum specimens, providing results that are independent of IgG concentration. At low antibody titers, endpoint methods are considered more accurate than methods that determine avidity at a single dilution. We determined that 6 months after the first MMR dose, mumps IgG avidity is high and generally sustained at avidity indices of 40 to 60%, reaching values of >80% in some individuals. Additionally, 4% (4/103) of individuals had avidity indices of ≤30% (low avidity) 2 years after vaccination. Inadequate mumps avidity maturation may be one factor influencing susceptibility to mumps virus infection among previously vaccinated or naturally infected individuals.

KEYWORDS IgG immunoglobulin, avidity immunoassay, measles-mumps-rubella vaccine, mumps, vaccine failure

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Despite excellent mumps vaccine seroconversion rates (first dose, 92 to 95%; second dose, 95 to 100%), mumps outbreaks occur with increasing frequency among highly vaccinated populations in the United States and internationally. The cause of recent outbreaks appears to be multifactorial, with low vaccine effectiveness (two doses are 88% effective [range, 79 to 95%]), congregation settings that facilitate transmission, waning neutralizing antibody, and strain variation playing a role (1–9). There is no established serologic correlate for mumps immune protection, and despite attempts to establish a threshold for protection, the amount and specificity of neutralizing antibody required for protection from clinical mumps disease remains unclear (10, 11). The avidity of mumps-specific IgG antibodies may also affect susceptibility to mumps virus infection in some individuals, but this has not been fully investigated (12–18).

IgG avidity indicates the overall binding strength of IgG antibodies to an antigen. Low-avidity IgG is synthesized after initial antigenic challenge and gradually matures into high-avidity IgG (19, 20). IgG avidity can be measured using enzyme immunoassays (EIAs) designed to include a protein-denaturing wash (21). Results are presented as the percent ratio of detected denaturant-resistant IgG over total untreated IgG (21), and they are referred to in this report as the avidity index (AI) for single-point methods and end titer avidity index (etAI) for endpoint titer methods. Determination of avidity by serum serial dilution to the endpoint (endpoint titer method) is considered the "gold standard" procedure because results are independent of IgG concentration (15, 21–23). The single-dilution method, however, is frequently used because it is faster and more economical than endpoint titer methods (12, 13, 15–18, 24–27). Using single-dilution methods, high-avidity mumps-specific IgG can be detected at symptom onset in acute-phase specimens from persons with a history of 1 or 2 measles, mumps, and rubella (MMR) vaccine doses and in mumps virus reinfections (12, 13, 15). AIs of >70% have been detected in response to mumps vaccine (16, 18) and wild-type mumps virus infection (12), as well as in secondary vaccine failure cases (15–17); however, in some persons exposed to wild-type or vaccine mumps virus in the distant past, AIs are at most around 50% (12–17), and AIs of <30% were described in samples collected 6 months and 20 years after receiving the second dose of mumps vaccine in the context of a mumps elimination setting (18). A correlation between mumps-specific IgG concentration and avidity has been reported (14, 17), although others have not observed this (18). The single-dilution method is limited by the linear range of the optical absorbance values (21). Results may be influenced by low IgG concentration in serum, which may lead to an underestimation of AIs; this is important when analyzing specimens collected from persons with waning immunity and IgG levels close to the lower limit of detection. The endpoint titer method has been recommended for measurement of avidity in specimens with very low IgG content; such a procedure is needed to more accurately assess the humoral response to mumps vaccine and mumps disease (15, 21, 22). The primary objectives of this study were to develop an endpoint titration IgG avidity assay for mumps and to measure mumps IgG avidity in vaccinated individuals. Secondary objectives were to describe mumps IgG avidity maturation following vaccination and to compare avidities resulting from the challenge of vaccine versus wild-type mumps virus antigen.

RESULTS

Sample selection from group A. To evaluate the performance of the mumps avidity assay, we selected samples from group A that had a paired prevaccine sample with an IgG-negative result. Out of 184 prevaccine specimens initially tested for mumps-specific IgG, 108 (58.7%) were negative, 29 (15.8%) were equivocal, and 47 (25.5%) were positive. Because of the high proportion of mumps IgG-positive results, we reevaluated the optical density ratio (ODR) threshold of the IgG EIA using receiver operating characteristic (ROC) analysis (see Fig. S1 and S2 in the supplemental material) and increased it from 1.1 to 1.5. Specimens selected for assay validation included 57 specimens collected 0.5 to 1 month (mean, 0.9 month) and 51 specimens collected 21 to 25 months (mean, 23 months) post-first MMR dose (post-MMR1). They were mumps

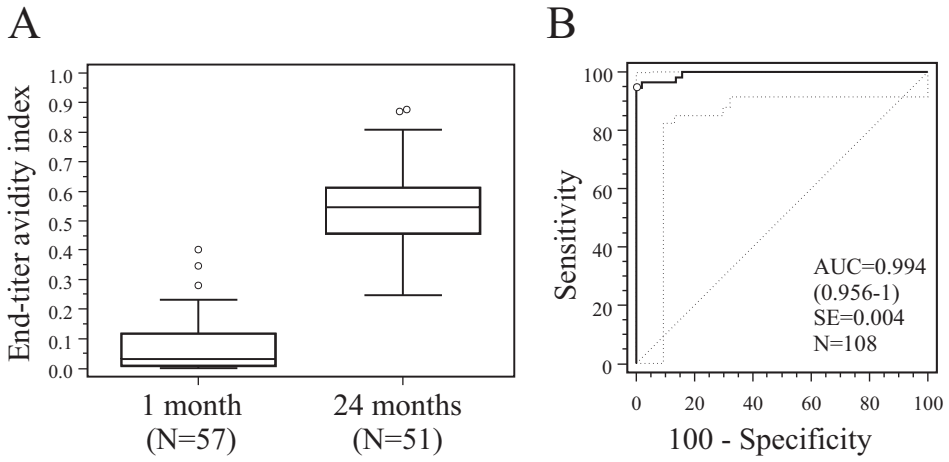

FIG 1 Diethylamine-based mumps IgG avidity assay. End titer avidity results were obtained from samples collected 1 month and 24 months after the first dose of mumps vaccine, administered at age 15 months. (A) Box-and-whisker analysis. Open symbols are values larger than the higher quartile plus 1.5 times the interquartile range. The low-avidity threshold is 30%. (B) Receiver operating characteristic curve. The diagonal line is an area under the curve (AUC) of 0.5, interpreted as a random guess. Ninety-five percent confidence intervals (CIs) are in parentheses and are plotted as dashed lines. The circle in the top left corner indicates the point with maximal accuracy.

IgG positive, and their cognate prevaccine specimens had ODRs below 1.5. The change from a negative mumps-specific IgG result to a positive result was taken as an indication of seroconversion.

Avidity assay development. Manufacturer's suggested wash buffer solutions containing 60 mM diethylamine (DEA) at various pH levels (11.70, 10.25, 10.00, 9.75, and 9.50) were evaluated using group A and C samples. It was found that three washes of 5 min each at room temperature using DEA at pH 10.00 ($\pm$0.1) had a minimal effect on the coating antigen. The solution was effective in eluting low-avidity IgG without eluting specific IgG with stronger avidity (data not shown). Solutions with a pH of $>$10 resulted in loss of optical density signal, suggesting that the pH influenced the stability of antigen on the microtiter plate matrix and the stability of antibody-antigen complexes. Therefore, three washes with 60 mM DEA adjusted to pH 10.00 ($\pm$0.1) were added to the protocol of the Zeus mumps IgG EIA after the serum incubation step; this procedure can be completed within 2.5 h.

Assay diagnostic performance and precision evaluation. The assay is highly accurate in distinguishing recent (0.5 to 1 month) from distant (21 to 25 months) immune responses to the first dose of mumps vaccine (Fig. 1 and Table 1). The within-device precision (S_T), or standard deviation estimate, was based on controls with etAIs of $<$30% (S_T = 6%), 40 to 70% (S_T = 8%), and $>$70% (S_T = 18%). Taking into account the assay variability, etAI values ranged from 0 to 100% (Table 1). The avidity threshold was established at an etAI of 30%, above which all samples are classified as high avidity. This threshold was selected by determining the threshold at which the sensitivity and specificity were highest (etAI of 23%), adding to it the S_T of 6% for the low-avidity control, and rounding up to 30%. At 30%, the sensitivity of the assay in detecting low-avidity IgG was 96.5% (95% confidence interval [CI], 87.9 to 99.6%). All samples collected within 1 month had an etAI of $\leq$30%, except for one sample collected at 3 weeks that had an etAI of 40% and one sample collected at l month that had an etAI of 35%. The specificity of the assay in detecting high-avidity IgG was estimated to be 92.2% (95% CI, 81.1 to 97.8%). All samples collected at 2 years had an etAI above the established threshold, except for four samples that were classified with a low-avidity result (Fig. 1 and 2).

Mumps IgG avidity maturation after the first dose of mumps vaccine. Sequential serum specimens were collected from 9 children after MMR1 (group A) and tested for mumps and measles IgG. All prevaccine samples were IgG negative for mumps and

TABLE 1 Mumps and measles IgG end titer avidity index values

Group and exposure	Group	Time after exposure	*n*[a]	Mean %	95% CI (%)[b]	Range (%)
Mumps vaccine	A	1 mo[c]	57	7	5–10	0–40
		2 yr[c,d]	51	54	50–58	25–88
		0.5–5.25 yr[e]	27	50[f]	46–54	30–71
Measles vaccine	A	0.5–5.25 yr[e]	27	80[f]	74–86	37–107
Mumps virus						
Noncases, unvaccinated	B	Distant[d]	29	62	58–66	46–86
Cases	D	Recent	27			
Unvaccinated		3–14 days	4	60	26–94	42–91
1 dose						
PVF[g]		Unknown onset date	1	17		
SVF[h]		2–4 days	3	52	18–86	40–67
2 doses						
PVF			0			
SVF		1–25 days	8	57	47–67	37–75
Unknown status						
Low avidity		Unknown onset date	3	24	10–37	18–29
High avidity		1–14 days	8	56	49–63	38–65

[a]*n* is the number of samples.
[b]CI, confidence interval.
[c]Samples analyzed to estimate the area under the curve.
[d]Means are significantly different ($P < 0.0001$).
[e]Samples analyzed to observe mumps IgG avidity maturation.
[f]Means are significantly different ($P = 0.0035$).
[g]PVF, primary vaccine failure, or failure to respond to the vaccine.
[h]SVF, secondary vaccine failure, or failure of vaccine resulting from waning immunity.

measles, and IgG-positive postvaccine samples were tested using both mumps and measles IgG avidity assays. During the first 6 months, mumps IgG avidity gradually increased, and a change from low- to high-IgG-avidity results was observed in all 9 sample sets: low etAIs were not obtained after 6 months with these sets of samples.

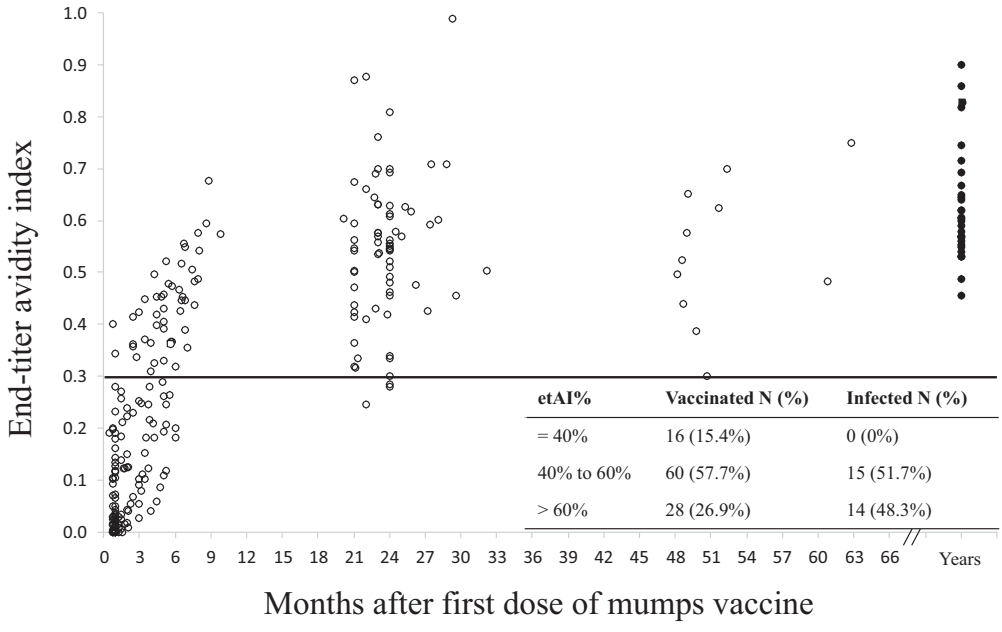

FIG 2 Mumps IgG avidity increases over time, and 6 months after the first dose of mumps vaccine, mumps-specific low-avidity IgG is generally not detected. Avidity was determined using an endpoint titer, diethylamine-based method. Open circles represent values from 15-month-old infants after their first mumps vaccine dose (group A; $n = 243$). Solid circles indicate values from unvaccinated adults who had mumps in the distant past (group B; $n = 29$). The inset table presents the number (*n*) and percentage of samples collected 6 months and later ($n = 104$) at various levels of end titer avidity index (etAI) within and around the 40 to 60% range of etAI values. The low-avidity threshold is indicated with a black horizontal line.

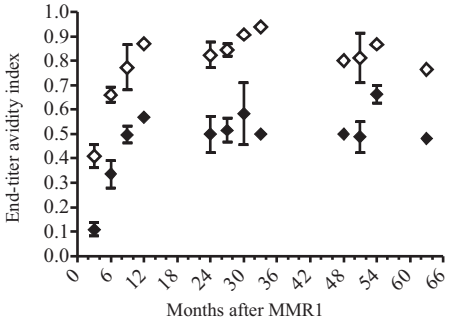

FIG 3 Mumps and measles IgG avidity maturation per the endpoint titer by the diethylamine-based method. Nine sets of 4 to 6 serum specimens were sequentially collected after the first dose of measles, mumps, and rubella (MMR1) vaccine. MMR2 was administered at about 6 months post-MMR1. Mean avidity values at every 3 months post-MMR1 are shown with error bars. Solid diamonds indicate mumps avidity. Open diamonds indicate measles avidity. The low-avidity threshold is 30% for both assays.

After 6 months, avidity stabilized at a mean etAI of 50% (Fig. 3 and Table 1), which was significantly different from the mean index of 80% obtained for measles IgG avidity ($P < 0.0001$). Overall, measles etAIs plateaued above 80%. In contrast, mumps etAIs plateaued at the 40 to 60% range and remained at this range at least 5 years after MMR1 (4.5 years after MMR2). This etAI range (40 to 60%) was also observed in 60 of 104 group A samples collected at different time points 6 months after MMR1; the remaining 44 samples spread above and below this 40 to 60% avidity range, with 6 samples having low-avidity results (Fig. 2).

Comparison of avidity after distant exposure to vaccine versus wild-type mumps virus. Testing of group B samples resulted in 51.7% or 15/29 having a mumps etAI in the 40 to 60% range; the remaining 14 samples tested above the etAI of 60% (Table 1 and Fig. 2). The mean etAI (62%) obtained from group B samples was significantly higher than the mean etAI (54%) obtained from a subset of 51 group A samples collected 2 years post-MMR1 ($P = 0.0035$) (Table 1 and Fig. 4).

Avidity in confirmed mumps cases collected in the United States, 2008 to 2013. A subset of 27 mumps IgM-positive convenience samples from group D were analyzed (Table 1). All but 4 cases had a high-avidity result, resulting from past immunization or infection. For the 23 past exposure cases, the mean mumps etAI was 57%, and the etAIs

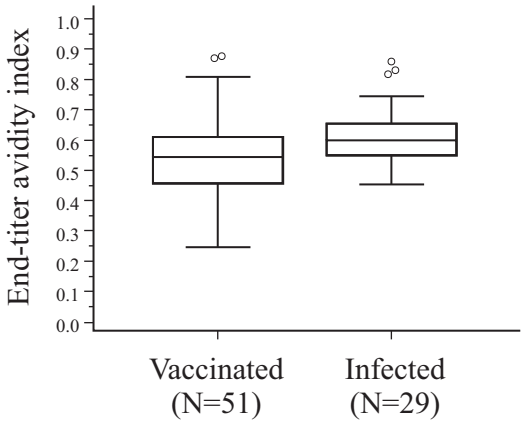

FIG 4 Comparison of mumps IgG avidity values after distant exposure to mumps virus for vaccine versus the wild type ($P < 0.05$). Box-and-whisker analysis is shown. Vaccinated group samples were collected 2 years after the first mumps vaccine dose. Infected group samples were collected from unvaccinated adults who had mumps in the distant past. Open symbols are values larger than the higher quartile plus 1.5 times the interquartile range. The low-avidity threshold is an end titer index of 30%.

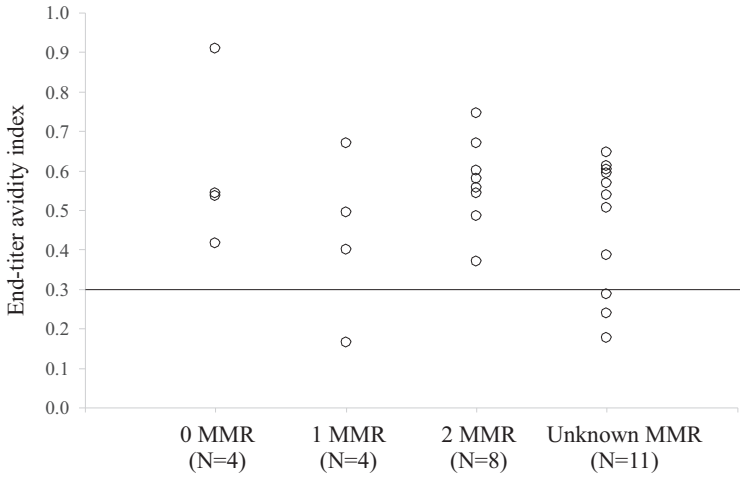

FIG 5 Mumps IgG avidity value distribution of 27 serum specimens collected from IgM-positive confirmed mumps cases from 2002 to 2013. The range of end titer avidity index values is 17 to 91%. Among the 23 with a high-avidity result, half of the avidity values (13/23) are at the midlevel range (40 to 60%).

mostly clustered in the 40 to 60% range, similar to groups A and B (Fig. 5 and Table 1). The highest value was found in an unvaccinated person.

DISCUSSION

A considerable increase in the number of mumps outbreaks, predominantly among twice-vaccinated populations, has prompted renewed interest in the role of mumps antibody avidity in the immune response to mumps vaccination. Previous reports using single-point avidity assays suggested that mumps IgG avidity maturation may fail to generate high-avidity antibodies in some individuals following vaccination (Urabe and/or Hoshino vaccine strains) (7, 15, 16). We developed and validated an endpoint titer mumps-specific IgG avidity assay to investigate the avidity of IgG antibodies generated in response to mumps virus antigens. The method is accurate (area under the curve [AUC] of >0.99), sensitive (97%), specific (92%), and rapid (<2.5 h) and clearly discriminates between primary mumps antigenic challenges (low avidity) and mature (high avidity) immune responses to MMR1. In agreement with Narita et al., we determined that mumps IgG etAIs increase from low (≤30%) to high (>30%) by 6 months post-MMR1 (15); for most individuals, mumps etAIs remain between 40 and 60%. This range of etAIs (40 to 60%) was also observed in 3 separate cohorts in this study and has been observed by others using single-dilution methods (13, 15). We found that IgG etAIs were lower and avidity maturation was slower to develop against the mumps component of MMR compared to the measles component of MMR, which by 9 months plateaued at etAIs of 80 to 100%. Mumps etAIs of ≥80% were observed in only 5% (4/84) of group A samples collected ≥9 months after MMR1, 10% (3/29) of group B adults, and 4% (1/23) of group D cases. Infection with wild-type mumps virus (group D) resulted in a recall memory B-cell response that resulted in production of specific IgG with etAIs in the range of 40 to 60% in all but 2 specimens (one was unvaccinated and one had 2 doses, both >70%). Maturation of mumps-specific IgG avidity to etAIs of >80% in response to mumps virus antigens, vaccine, and the wild type appears to be variable and not completely achieved by every individual.

Our conclusions contradict those of Kontio et al. (18), who reported that mumps IgG avidity wanes after vaccination, becoming low avidity as soon as 6 months after MMR2 (mean avidity index of 21%) and significantly lower 20 years later (mean avidity index of 16%). In their study, results for avidity maturation after MMR1 and before collection at 6 months post-MMR2 were not reported. We did not observe low-avidity indices in any of the unvaccinated, naturally infected adults or in samples collected ≥9 months post-MMR1, except for the 4 samples discussed below. We did not observe waning of

IgG avidity at 1.5 or 4.5 years after MMR2 (2 and 5 years after MMR1). However, our results were consistent with those of Kontio et al. (18) in that mumps IgG avidity after natural disease is higher than after vaccination. We detected significantly higher etAIs in unvaccinated adults (group B) compared to vaccinated children (group A), but in contrast to the low-avidity results reported by Kontio et al., we present data that support the maturation of mumps avidity to at least a 40 to 60% avidity range, classifying these samples as high avidity. In our study, none of the naturally infected individuals had low-avidity results, and 4/82 in the vaccinated children with samples collected 1.5 years after MMR2 had low-avidity results. It is unlikely that these 4 low-avidity results were due to mumps virus infections in individuals with two-dose primary vaccine failure, due to the low mumps virus circulation at the time (28). Unfortunately, paired specimens collected weeks after MMR1 were not available to determine if there had been waning of IgG avidity leading to a low-avidity result or if avidity was initially low and never progressed to higher-avidity levels. It is possible that mumps-specific IgG avidity maturation stalled at a low-avidity level, similar to individuals who did not initiate IgG maturation even after two doses of varicella vaccine (29) or after multiple infections with *Plasmodium falciparum* (30).

Our results are consistent with those of Narita and Park (15, 16), who concluded that in general there is a failure to generate high-avidity mumps IgG antibodies (for instance, >70%), based on observation of avidity indices close to borderline levels (high avidity of >31%). In our report, the higher etAIs of 40 to 60% that were measured concur with results obtained by others after mumps virus infection (13, 24) and after vaccination with various mumps vaccine strains (Urabe [7, 15, 16], Leningrad-3 [12], Hoshino [7, 14, 16], and Jeryl-Lynn [17]). Similar IgG avidity indices (40 to 60%) have been observed in children after respiratory syncytial virus infection and in healthy individuals after vaccination with varicella and rubella vaccines and are not unique to the mumps immune response (29, 31, 32). It would be expected that if maturation of mumps IgG avidity fully progressed to completion after vaccination or infection, avidity indices would be as high (>70%) as those observed after exposure to measles virus, human papillomavirus, or cytomegalovirus antigens (this report and references 14, 18, 33, and 34).

IgG avidity maturation is a sophisticated and regulated antigen-dependent process that involves T-cell activation, B-cell somatic hypermutation of IgG genes, and selection of high-affinity clones (19, 20, 35). For instance, insufficient Toll-like receptor stimulation in B cells prevented IgG avidity maturation in response to a formalin-inactivated respiratory syncytial vaccine, which resulted in enhanced respiratory disease (36). It is intriguing that the number of mumps-specific memory B cells elicited in response to second and third doses of MMR vaccine was found to be lower than the corresponding measles and rubella responses (37). The connection between low memory B-cell response and the observed partial maturation of mumps IgG avidity is unclear.

Our new endpoint titer assay is a useful tool to measure the avidity of mumps IgG for research purposes. Its advantages are 2-fold. First, the assay was intentionally designed to detect low levels of IgG by starting the serum dilution series at 1:21, the original dilution recommended in the kit's instructions. This dilution scheme facilitates the determination of mumps IgG avidity in specimens with low mumps-specific IgG titers, such as those collected from young adults 10 to 20 years postvaccination. This approach allowed us to measure avidity in samples with low IgG concentrations, collected only weeks after MMR1. The dilution scheme also works well for samples with high IgG titers (data not shown). Second, calculation of etAI using the optical density values obtained at endpoint titers alleviates concerns about the effect of IgG concentration on avidity results (14, 17, 18). Additionally, a strength of this study is the use of group A specimens to validate the assay because they were collected from originally unvaccinated young children and presumed to be immunologically naive to mumps virus antigens.

There are some limitations to our mumps IgG avidity assay. First, serum samples must have sufficient mumps-specific IgG to be detected by the Zeus mumps IgG assay.

We observed lower than expected specificity on IgG detection, also observed by others (38). To develop this mumps avidity assay, we raised the Zeus assay threshold to 1.5, which may increase the number of specimens classified as IgG negative. Second, the avidity results obtained are intrinsically related to presentation of epitopes on the antigen coated on the plates. The coated antigen is partially purified cell lysate containing the Enders strain (per communication with manufacturer), which is a genotype A virus that is closely related to the Jeryl-Lynn vaccine strain (39). The actual proportion of each mumps virus protein coated on the plate is unknown, but the most abundant protein is likely to be the nonneutralizing nucleoprotein, produced in higher quantities in viruses of the *Paramyxoviridae* family; this appears to be the most immunogenic and abundant of the proteins (38, 40). Therefore, the results are not necessarily representative of the avidity of mumps-neutralizing IgG antibodies nor associated with protection. However, specificity for the nucleoprotein is also likely to occur in mumps avidity assays that use other platforms (15). Third, cross-reactivity of the Zeus mumps IgG EIA with parainfluenza virus-specific antibodies was not studied in this or previous studies (15, 41–43). Additionally, samples from unvaccinated mumps acute cases were not used to evaluate the assay because of the possibility of detecting high-avidity IgG in individuals with prior asymptomatic mumps (13, 24).

In summary, we describe a mumps-specific IgG avidity assay using the endpoint titer method, which can be used to study the avidity of mumps IgG antibodies in high- and low-vaccine-coverage settings experiencing mumps outbreaks. We determined that mumps IgG avidity matures over the course of several months and is generally sustained at a midlevel range of etAIs of 40 to 60%, but can reach etAIs of >80% in some individuals. Inadequate mumps IgG avidity maturation may be one important factor in susceptibility to mumps virus infection among previously vaccinated or naturally infected individuals and should be studied further.

MATERIALS AND METHODS

Study specimens. Serum specimens from 5 groups described below were collected after necessary informed consent was obtained for all study participants and under the approval of the Centers for Disease Control and Prevention (CDC) Institutional Review Board. All specimens tested for avidity contained mumps-specific IgG, as detected by the Zeus mumps IgG enzyme immunoassay (EIA [Alere, Inc., Waltham, MA]). Serum samples were obtained from 5 cohorts as described below.

Group A consisted of 243 mumps-IgG positive serum specimens from a previously described archived collection (38). These specimens were collected during 1990 to 1997 from healthy children who had received their first dose of MMR (MMR1) vaccine (MMR II; Merck & Co., Inc.) at age 14 to 18 months and a second dose of MMR (MMR2) 5 to 6 months later. Because there was extremely low mumps virus circulation at the time (28), it was assumed that the children had not had mumps prior to MMR1 and that they were immunologically naive to mumps antigens. It was also assumed that the children did not have circulating maternal IgG antibodies (44). In relation to MMR1, sera were collected from these children at four visits resulting in the following overlapping time frames: 0.5 to 6 months ($n = 107$), 3 to 8 months ($n = 34$), 5 to 10 months ($n = 20$), 1.7 to 2.7 years ($n = 71$), and 4.0 to 5.3 years ($n = 11$) postvaccination. The mean age of participants at the time of study enrollment was 15.1 months (range, 13 to 31 months); 48.5% of participants were female. Final specimen selection is discussed in Results.

Group B included 37 specimens drawn from healthy adult donors born before 1957 who reported they had mumps disease in the distant past and had never been vaccinated with the mumps vaccine. The assumption was that specimens from group B would have mumps-specific, high-avidity IgG from natural exposure to mumps virus infection at least 40 years earlier.

Group C consisted of 8 specimens obtained from healthy adult donors vaccinated with MMR in the distant past; these were presumed to contain high-avidity IgG.

Group D comprised 82 specimens received at CDC for routine mumps diagnostic testing. Specimens were positive in the CDC IgM capture assay (45) and were from individuals who had received 0, 1, or 2 doses of MMR or whose vaccination status was unknown.

Group E consisted of 84 samples collected before mumps vaccination from children ages 15 to 23 months with upper respiratory symptoms who attended immunization clinics in Atlanta, GA, from February 1992 to April 1993 (46).

Assay development. No gold standard method for measuring mumps IgG avidity exists. We assumed that unvaccinated persons receiving MMR1 would have a primary immune response resulting in IgG seroconversion between paired pre- and postvaccine samples collected within 6 months following vaccination (15). Therefore, four convenience serum specimens with demonstrated IgG seroconversion were selected from group A with collection at 2 months ($n = 3$) and 4 months ($n = 1$) post-MMR1 postvaccination. Next, we assumed that the avidity assay would detect a mature mumps-specific IgG response in specimens collected from individuals previously vaccinated with mumps vaccine or previ-

ously infected by the mumps virus (17 to 50 years earlier) (15). Therefore, 15 convenience serum specimens were selected from group B ($n = 8$) and group C ($n = 7$).

These 19 specimens were used to test the effect of diethylamine (DEA) on antigen and antibody binding as previously described (47). Specifically, specimens were used to determine the effect of the wash buffer and 60 mM DEA solutions at pH values ranging from 8 to 11. These solutions were used to directly wash mumps antigen coated on microtiter plates or to elute serum IgG from IgG-antigen immune complexes that were formed during the serum incubation step.

Evaluation of precision. Standard EP05-A2 from the Clinical and Laboratory Standards Institute was followed to evaluate assay precision and calculate the within-device precision standard deviation estimate (S_T) (48). S_T is a global precision estimate that considers estimates of repeatability and between-day and between-run standard deviations of the assay. We randomized the samples and tested them in 20 separate runs. Preliminary thresholds of avidity indices were established during a pilot study by testing 10 sets of paired samples from group A that were collected in a 1- to 29-month interval after MMR1 administration. For the precision experiment, two operators tested three controls at low, mid, and high levels of etAIs. Ranges calculated from the mean etAI of an avidity control $\pm 2\times S_T$ indicated the quality control of the runs.

Avidity assay for mumps virus-specific IgG antibodies. The Zeus mumps IgG EIA protocol was adapted for use with the denaturant agent DEA, as previously described for a measles avidity assay (47). This EIA uses whole mumps virus extracts (Enders strain) as the detecting antigen. Briefly, serum avidity controls (20 μl) and test samples (20 μl) were diluted in Zeus serum diluent (190 μl) and were 10-fold serially diluted in two single-well dilution series. One dilution series started at 1:21 and was washed with Zeus wash buffer (WB) after serum incubation. The other dilution series started at 1:10.5 and was washed with 60 mM DEA solution in WB adjusted to pH 10.00 ($\pm$ 0.1) with 1 M hydrochloric acid after serum incubation. For both series, three washes for 5 min each at 20 to 25°C were performed. This was followed by five washes with WB without soaking. All other steps of the assay were performed as described by the manufacturer. Specifically, serum and conjugate were incubated for 20 min, and the chromogen was incubated for 10 min, all at 20 to 25°Cs. A run was accepted per Zeus's quality control criteria and per avidity control criteria, which were derived from the precision experiment data (see "Precision evaluation" above). Results were calculated using a threshold based on an optical density ratio (ODR) of 1.5 and expressed as end titer avidity index (etAI) percentages as previously described (47, 49). The results were classified as low avidity if the etAI was $\leq$30% and high avidity if the etAI was $>$30%. Samples at 1:10.5 dilution with undetectable IgG after DEA treatment were classified as low avidity.

Assay validation. A subset of 108 unpaired specimens from group A were analyzed. They were collected approximately 1 month ($n = 57$) or 2 years ($n = 51$) after MMR1. For each of these specimens, there was an available paired prevaccine serum that was tested for mumps-specific IgG content.

Avidity maturation. Nine sets of 4 to 6 paired specimens ($n = 50$) from group A were studied. The intervals between MMR1 and sample collection were less than 1 year (1 to 10 months; $n = 29$), 2 years (23 to 32 months; $n = 10$), 4 years (48 to 52 months; $n = 9$), and 5 years (61 to 62 months; $n = 2$). These sets were tested by the mumps IgG avidity assay and by a previously described endpoint titration measles IgG avidity assay (47). A second subset of samples from group A collected at 1.25 to 6 months ($n = 50$) were tested by the mumps avidity assay, and the results were added to the analysis.

Statistical analyses. Receiver operating characteristic curve (ROC) analysis was used to reevaluate the ODR threshold of the Zeus mumps IgG EIA. First, 42 group A prevaccine specimens known to be negative for mumps IgM and negative by plaque reduction neutralization (PRN [titers of $<$1:8]) were compared to 55 group D specimens collected from confirmed mumps cases that were positive for mumps IgM and had PRN titers of $\geq$1:31 (10, 38, 45). The mumps PRN test was used as the gold standard for detection of mumps-specific antibodies, because it is a functional assay that detects neutralizing antibodies and is considered the most predictable surrogate marker of protective immunity to mumps. A second analysis compared 84 group E specimens and 84 group A, B, and C specimens. To evaluate the performance of the mumps avidity assay, specimens described under "Assay validation" were analyzed using ROC analysis to estimate assay accuracy, set the etAI threshold, and estimate the corresponding sensitivity and specificity (50). The null hypothesis for sample size calculation for both ROC analyses was an area under the curve (AUC) of $\leq$0.75 (moderate diagnostic accuracy) and standard error of the AUC of $\leq$5% (50, 51). The *t* test (assuming equal variances) and Welch test (assuming unequal variances) were used for comparisons of the means (independent samples). Statistical analyses and graphs were performed with MedCalc for Windows, version 16.2.1 (MedCalc Software, Belgium), and Prism (GraphPad Software, Inc., La Jolla, CA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00320-18.

FIG S1, EPS file, 2.2 MB.

FIG S2, PDF file, 0.4 MB.

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