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Mumps Virus-Specific Antibody Titers from Pre-Vaccine Era Sera: Comparison of the Plaque Reduction Neutralization Assay and Enzyme Immunoassays

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Mumps virus-neutralizing antibodies are believed to be the most predictable surrogate marker of protective immunity. However, assays used to detect neutralizing antibodies, such as the plaque reduction neutralization (PRN) assay, are labor- and time-intensive and consequently are often supplanted by the more rapid and inexpensive enzyme immunoassay (EIA) technique. For virus infections for which international antibody standards exist and are bridged to clinical studies of protection (e.g., measles and rubella), the EIA has been successfully used to determine immune surrogate endpoints, yet no such international reference exists for mumps serology. Since both virus-neutralizing and nonneutralizing antibodies are measured in the EIA, in the absence of a mumps serological standard, the EIA may be prone to yielding false-positive results when utilized for assessing surrogate markers of protective immunity. Moreover, since mumps virus-specific antibody titers are generally low in comparison to antibody levels induced by other viruses and EIA procedures often employ relatively high serum dilution factors, the EIA may be prone to yielding false-negative results. To examine these issues, a PRN assay and two commercially available EIA kits were used to evaluate wild-type mumps virus serological responses in human serum samples from the pre-mumps vaccine era. Our results indicate that the PRN assay is a more sensitive and specific method of measuring serological responses to wild-type mumps virus.

Protective efficacy field studies have shown that mumps virus-neutralizing antibody titers as low as 1:2 provide protection against mumps (10, 30–32). Accordingly, virus neutralization assays, such as the plaque reduction neutralization (PRN) assay, have long been the "gold standard" in determining the presence of protective immunity against mumps virus infection (3, 24, 32). The PRN assay measures the serum dilution (titer) capable of preventing 50% of plaque formation by mumps virus in cell cultures. Although virus neutralization assays may be the most predictive technique for assessing protective immunity, these assays are often not standardized and are extremely skilled labor- and time-intensive, making examining large numbers of human sera by PRN assay difficult. In contrast, the enzyme immunoassay (EIA) technique is simpler to perform and provides rapid, quantitative results and, thus, is the most widely used technique in clinical serology testing. However, since the EIA does not distinguish neutralizing from nonneutralizing antibodies, this assay may be prone to yielding false-positive results in the context of assessing protective immunity. In many cases, e.g., measles and rubella serology, an international standard referenced to a protective serum titer is used within the context of the EIA to provide a reasonable immune surrogate marker of protection. No such mumps standard exists. Further, since mumps virus-neutralizing antibody titers are often low (less than or equal to 1:8) and EIA procedures require initial serum dilutions as high as 1:100, these assays may theoretically be insensitive to detecting protective but low levels of mumps virus-specific antibody, i.e., yielding false-negative results. Thus, the purpose of this study was to assess the correlation of mumps serologies directed to the same strain of wild-type mumps virus as measured in the PRN assay and the EIA, e.g., the extent of EIA false-positive and false-negative rates as measured against a standardized PRN assay.

As a rule, immunity following wild-type mumps virus infection confers lifelong protection against subsequent mumps disease. While mumps vaccination attempts to emulate immune responses to natural infection without producing the serious consequences of wild-type disease, occasionally problems have arisen with inadequate induction of antibody directed against neutralizing epitopes (primary vaccine failure) and/or waning immunity (secondary vaccine failure) (2, 4, 5, 9, 25, 33). Accordingly, studies have shown that immunization results in lower levels of neutralizing antibody than can be seen following natural mumps virus infection (1, 6, 26, 29, 32). Notably, most mumps serological evaluations utilizing the EIA technique have been performed subsequent to the institution of widespread mumps vaccination; thus, little is known about the performance of EIA-measured mumps serology in the setting of wild-type infection and serological response and about the correlation of EIA mumps serology titers and protection from mumps. Thus, for this study, we compared the performance of the PRN assay and that of the EIA on pre-vaccine era sera, e.g., measured serological responses reflected infection with wild-type mumps virus.

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TABLE 1. Results of PRN and EIA testing of 74 pre-vaccine era serum samples

Serum no.	PRN titer[b]	EIA result[a]		Serum no.	PRN titer[b]	EIA result[a]	
		Wampole	IBL			Wampole	IBL
1	1:128	+	+	38	1:4	+	+
2	1:128	+	+	39	1:4	+	+
3	1:128	+	+	40	1:4	+	+
4	1:128	+	+	41	1:4	+	
5	1:128	+	+	42	1:4	−	+
6	1:64	+	+	43	1:4	−	−
7	1:32	+	+	44	1:4	−	−
8	1:32	+	+	45	1:4	−	−
9	1:32	+	+	46	1:4	−	−
10	1:32	+	+	47	1:4	−	−
11	1:32	+	+	48	<1:4	−	+
12	1:32	+	+	49	<1:4	+	+
13	1:16	+	+	50	<1:4	−	−
14	1:16	+	+	51	<1:4	−	−
15	1:16	+	+	52	<1:4	−	−
16	1:16	+	+	53	<1:4	−	−
17	1:16	+	+	54	<1:4	−	−
18	1:16	+	+	55	<1:4	−	−
19	1:16	+	+	56	<1:4	−	−
20	1:16	+	+	57	<1:4	−	−
21	1:16	+	+	58	<1:4	−	−
22	1:16	+	−	59	<1:4	−	−
23	1:16	−	−	60	<1:4	−	−
24	1:16	−	−	61	<1:4	−	−
25	1:8	−	−	62	<1:4	−	−
26	1:8	−	−	63	<1:4	−	−
27	1:8	+	+	64	<1:4	−	−
28	1:8	+	+	65	<1:4	−	−
29	1:8	+	+	66	<1:4	−	−
30	1:8	+	+	67	<1:4	−	−
31	1:8	+	+	68	<1:4	−	−
32	1:8	+	+	69	<1:4	−	−
33	1:8	−	+	70	<1:4	−	−
34	1:8	−	−	71	<1:4	−	−
35	1:8	−	−	72	<1:4	−	−
36	1:8	−	−	73	<1:4	−	−
37	1:8	+	−	74	<1:4	−	−

[a] EIA, enzyme immunoassay; +, positive; −, negative.
[b] A titer of <1:4 was considered negative.

Sera. Sera were obtained prior to 1964 (a time prior to the development and use of mumps vaccines) from 74 adolescent women in the United States. All sera were stored at −20°C or below at the time of the draw and were not thawed until the initiation of the present study.

PRN assay. Neutralizing anti-mumps virus antibody titers were determined by PRN assay. Briefly, sera were thawed at room temperature and heated at 56°C for 45 min to inactivate complement. Twofold serial dilutions of heat-inactivated serum (or medium alone as a negative control) were mixed with equal volumes of approximately 30 PFU of the mumps virus Enders strain (ATCC VR-106) to give a final serum dilution range of 1:4 to 1:128. Serum-virus mixtures were incubated at 37°C with 5% CO_2 for 1 h and then placed on Vero cell monolayers in 24-well plates and incubated for 1 h at 37°C with 5% CO_2. The virus-serum mixture was removed by aspiration, and cell monolayers were rinsed with minimum essential medium immediately before being covered with 0.75% agar (Nobel) in 2× minimum essential medium (Quality Biological, Gaithersburg, MD) supplemented with 10% fetal bovine serum. Plates were then incubated at 37°C with 5% CO_2 for 5 days. A second layer of agar containing 0.01% neutral red (Quality Biological) was added and incubated overnight to visualize plaques produced by remaining infectious virus. For each serum sample, the neutralizing antibody titer was determined as the highest dilution of serum capable of reducing the number of virus plaques by 50% or greater compared to control values (virus incubated with negative control serum). The cutoff for seropositivity was a neutralizing antibody dilution greater than or equal to 1:4, the minimal titer observable in this assay. While neutralizing antibody dilutions of 1:2 have been found to be protective, such concentrated sera have been found to have nonspecific antiviral activity and were therefore not assessed here (7, 27).

EIA. All sera were also tested with IBL (Hamburg, Germany) and Wampole Laboratories (Cranbury, New Jersey) mumps virus immunoglobulin G (IgG) EIA kits according to the manufacturers' instructions. Both manufacturers' assays for mumps virus IgG are based on capturing virus-specific human IgG on a preparation of purified virus antigen (derived from the mumps virus Enders strain) immobilized on plastic wells. In the Wampole assay, sera are diluted 1:21, whereas for

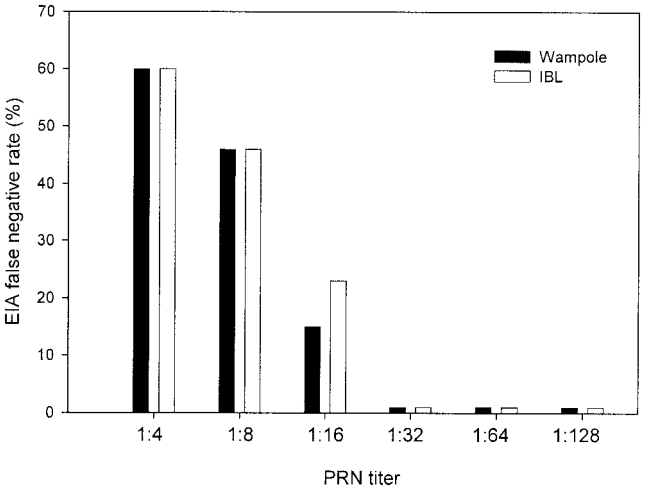

FIG. 1. Inverse relationship between the PRN titer cutoff for a positive response and the EIA false-negative rate.

TABLE 2. Results of EIA testing of 10 serum samples possessing measurable PRN titers before and after dilution, indicating that the EIA may be relatively insensitive to low levels of antibody

Serum sample no.	Result under indicated condition[a]					
	No predilution[b]			Prediluted to low PRN titer[c]		
	PRN titer	EIA result		PRN titer	EIA result	
		Wampole	IBL		Wampole	IBL
1	1:128	+	+	8	−	−
2	1:128	+	+	4	−	−
3	1:128	+	+	8	−	−
6	1:64	+	+	8	−	−
7	1:32	+	+	8	−	+
8	1:32	+	+	4	+	+
9	1:32	+	+	4	−	−
10	1:32	+	+	8	−	−
11	1:32	+	+	4	−	+
12	1:32	+	+	8	−	−

[a] EIA, enzyme immunoassay; +, positive; −, negative.
[b] Sera were tested per standard PRN and EIA protocols.
[c] Sera were diluted to achieve nominal levels of neutralizing antibody prior to testing in the PRN and EIA tests.

the IBL assay, sera are diluted 1:101. For both assays, following incubation with sera, wells were washed three times in phosphate-buffered saline and incubated with anti-human IgG conjugated to horseradish peroxidase. After being washed, wells were incubated with tetramethylbenzidine substrate solution. The reaction was stopped by addition of H_2SO_4. Plates were then read on an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm using a reference wavelength of 650 nm. All reagents used were provided with the EIA kits. Absorbance value cutoffs and interpretation of results were carried out according to the manufacturer's instructions.

Additional EIA testing was carried out on a subset of 10 serum samples with neutralization dilutions greater than or equal to 1:32 that were diluted in phosphate-buffered saline to achieve PRN dilutions of 1:4 and 1:8.

Among the 74 pre-vaccine era serum samples, 47 (64%) were seropositive by PRN (Table 1). Of these 47 PRN-positive samples, 33 tested positive in the Wampole EIA and 32 tested positive in the IBL EIA. Thus, measured against the PRN assay, the sensitivities of the Wampole and IBL EIAs were 70% and 68%, respectively, translating to false-negative rates of 30% and 32%, respectively. That the EIAs apparently did not react with sera found to contain neutralizing antibody by PRN was intriguing, especially in view of the fact that the EIA is capable of detecting a broader spectrum of antibodies than virus neutralization assays (4, 6, 11, 12, 28). One possible explanation for EIA false negativity may be that the EIA is relatively insensitive to low levels of antibody. This hypothesis is supported by the observation that the EIA false-negative rate dramatically decreases as the PRN titer increases (Fig. 1). An explanation for the inability of the EIA to detect low levels of antibodies may lie with the fact that the initial serum dilution steps in the Wampole and IBL assays (1:21 and 1:101, respectively) are significantly greater than that of the PRN assay (1:4). Thus, one could postulate that the initial serum dilution step in the EIA was sufficiently high to dilute the low-PRN-titer sera to a dilution below the minimum detection capacity of the EIA. To test the hypothesis that low levels of virus antibody detectable by PRN are lost upon preparation for use in EIA, contributing to EIA false negativity, all 10 of the serum samples that had PRN titers greater than or equal to 1:32 were diluted to achieve PRN titers of 1:4 to 1:8. These diluted serum samples were then retested in the EIA tests. As shown in Table 2, postdilution, the majority of the serum samples tested negative in these assays (yet continued to be reactive in the PRN assay), i.e., 9 of the 10 samples that tested positive (when undiluted) in the Wampole EIA and 7 of the 10 samples that tested positive (when undiluted) in the IBL EIA tested negative. Thus, it appears that low levels of virus antibody detectable by PRN are lost during the dilution steps required in preparation for testing in EIA. Notably, EIA insensitivity to low levels of neutralizing antibody has also been reported for measles virus antibody (19). This hypothesis alone does not account for all observed instances of EIA insensitivity, since some of the retested sera in the present study remained EIA positive despite dilution. This might indicate, at least for these samples, that nonneutralizing antibodies may exist in higher concentrations than neutralizing antibodies. Other factors that may contribute to negative EIA findings include the ability of the PRN assay to detect all classes of mumps virus-specific immunoglobulins (whereas the EIA kits measure only IgG antibodies) and loss of conformational epitopes in the EIA format. In addition, it should be pointed out that nonspecific reactivity has been reported in the PRN assay when concentrated serum, defined as having a dilution less than or equal to 1:4, was used (7, 27). Thus, an alternative explanation for some of the PRN assay-positive/EIA-negative results could be nonspecific virus neutralization in the PRN assay. Notably, however, of the 74 serum samples tested, only two (numbers 41 and 42) were PRN assay positive/EIA negative at a 1:4 dilution (the most concentrated dilution of serum used). Thus, should nonspecific reactivity occur in the PRN assay at a 1:4 dilution of serum, the effect on this study is negligible.

In terms of EIA specificity, of the 27 serum samples testing negative by PRN assay, 26 were negative in the Wampole EIA

and 25 were negative in the IBL EIA. Thus, relative to the PRN assay, the specificity of the Wampole EIA was 96% and that of the IBL EIA was 93%. This translates to Wampole and IBL EIA false-positive rates of 4% and 7%, respectively. These PRN assay-negative/EIA-positive sera, although deemed to represent EIA false positivity, may nonetheless contain nonneutralizing anti-mumps virus antibodies (12). This is likely, since the preparations that coat the EIA plates are predominated by virus proteins containing numerous nonneutralizing mumps virus epitopes (14, 15, 23). Perhaps the development of EIA plates coated with mumps proteins known to be associated with virus neutralization (e.g., HN and F proteins) may provide for a better bridge between the two assays. Another possible explanation for the EIA false-positive results could be the presence of antibody in these particular samples directed against other related viruses, resulting in a positive reaction by EIA but not sufficiently specific to result in virus neutralization. Indeed, a number of studies have found cross-reactivity to parainfluenza viruses 2 and 3 in mumps virus EIAs but not in mumps virus neutralization assays (4, 6, 8, 13, 16).

In comparing the PRN assay results to the EIA results, one must also be mindful of inherent vagaries of any interassay comparison whose cause cannot be identified. This is most plainly evident in an inter-EIA comparison of the two kits used here. Although both the Wampole and IBL EIA kits are very similar in apparent design and use, e.g., employing similar preparations of the mumps virus Enders strain, similar procedures, and utilization of the same enzyme-substrate reaction, 8% (6/74) of the serum samples tested in both EIA kits yielded discordant results. Of note, the difference in sample dilution between these two assays (1:21 for Wampole and 1:101 for IBL) does not account for this discordance, since three of the six serum samples were Wampole positive/IBL negative and the other three were Wampole negative/IBL positive. Thus, some of the discordance in test results between the PRN assay and the EIA is probably attributable to intrinsic assay variability.

The fact that pre-vaccine era sera were used adds additional significance to this study. Because the EIA technique was not available during the pre-vaccine era, most of our knowledge of EIA performance in evaluating mumps immune responses is based on testing of vaccinated populations. However, unlike even low-titer immune responses to natural infection, serological responses engendered by vaccination cannot be assumed to be protective. For example, despite EIA determinations of high seroconversion rates following vaccination with the Rubini vaccine strain (17, 22), this particular vaccine afforded virtually no protection against mumps disease (18, 20, 21). Thus, for evaluating an assay's ability to provide information about protective immunity, use of sera from cases of natural infection may provide better bridging to efficacy endpoints. Using such sera, our data indicate that the PRN assay was a more sensitive and specific method of measuring serological responses to mumps virus than the EIA.

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