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Estimates of Mumps Seroprevalence May Be Influenced by Antibody Specificity and Serologic Method

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Neutralizing antibodies are assumed to be essential for protection against mumps virus infection, but their measurement is labor- and time-intensive. For this reason, enzyme-linked immunosorbent assays (ELISAs) are typically used to measure mumps-specific IgG levels. However, since there is poor correlation between mumps neutralization titers and ELISAs that measure the presence of mumps-specific IgG levels, ELISAs that better correlate with neutralization are needed. To address this issue, we measured mumps antibody levels by plaque reduction neutralization, by a commercial ELISA (whole-virus antigen), and by ELISAs specific for the mumps nucleoprotein and hemagglutinin. The results indicate that differences in the antibody response to the individual mumps proteins could partially explain the lack of correlation among various serologic tests. Furthermore, the data indicate that some seropositive individuals have low levels of neutralizing antibody. If neutralizing antibody is important for protection, this suggests that previous estimates of immunity based on whole-virus ELISAs may be overstated.

Infection with mumps paramyxovirus used to be a common childhood occurrence prior to the era of widespread vaccination. Historically, mumps was a leading cause of hearing loss, and it can cause other serious complications such as parotitis, orchitis, meningitis, and pancreatitis. Following routine administration of measles-mumps-rubella (MMR) vaccine, the number of mumps cases in the United States dropped from tens of thousands per year to less than 300 on average (1). From 1994 to 2005, it appeared as if mumps would be effectively eliminated from the United States. However, two outbreaks occurred during 2006 to 2007 and 2009 to 2010 that together affected over 8,000 individuals, most of whom had previously received two doses of the MMR vaccine (1–4). Mumps outbreaks among two-dose vaccine recipients are not unique to the United States or to the Jeryl Lynn vaccine strain (the only licensed mumps vaccine in the United States). In fact, mumps outbreaks among vaccinated populations have occurred in numerous countries, some of which utilize different strains of vaccine virus (5–12).

It is not clear why mumps outbreaks are sometimes sustained in highly vaccinated populations, but there are probably several contributing factors. Although antigen drift has been hypothesized as a logical culprit, it has been repeatedly shown that the most divergent strains can be neutralized *in vitro* with only slight variations in serum titers (typically 2- to 8-fold) (13–15). The observation that naturally acquired wild-type mumps infection does not necessarily confer life-long protection (16, 17) suggests that the immune memory to mumps may be inherently unreliable and that breakthrough infection among certain vaccinated individuals could arguably be expected. Furthermore, estimates of mumps vaccine efficacy among two-dose recipients vary, but most are approximately 90% (10, 18). The consequences of less-than-optimal vaccine effectiveness could be compounded by incomplete vaccination coverage, waning immunity, and a lack of asymptomatic natural boosting due to substantially reduced endemic disease. A common element in some recent outbreaks has been the presence of environmental conditions that foster frequent, high-intensity exposures, such as those found in dormitories (4, 19, 20). In light of these observations, it has been suggested that protection against classic mumps symptoms may not be an all-or-none phenomenon but may be a continuum that is a function of the amount of virus an individual is exposed to and their personal level of immunity (19, 20).

To complicate matters further, it is not known what (if any) specific humoral or cellular components of the anti-mumps immune response may provide reliable assurance of protection against symptomatic wild-type mumps infection (18). It is assumed that neutralizing antibody is likely essential for protection, while cell-mediated immunity (CMI) is perhaps necessary but is probably not sufficient (21). Early vaccine efficacy studies suggested that an *in vitro* plaque reduction neutralization (PRN) antibody titer as low as 1:2 to 1:8 might be protective (22–24). However, these studies were done when circulating wild-type virus was still common and subclinical infections among some vaccinated individuals were highly suspected to have occurred based on unexpected rises in antibody titers (23). As a result, it was not possible to accurately predict the long-term persistence of antibodies or the long-term protective efficacy of the vaccine based on these data. A recent attempt to define a protective threshold for neutralizing antibody titer was inconclusive, partly due to the use of convenience samples that were not collected at ideal times and the inclusion of a limited number of cases (20). Nevertheless, the data indicate that case patients had lower neutralizing antibody titers (specific for the Jeryl Lynn strain) than nonpatients who had no known mumps exposure ($P = 0.023$). Relative to nonpatients, proportionately more case patients had neutralizing titers of <31, and antibody titers among case patients were also lower than those of nonpatients when measured by enzyme-linked immunosorbent assay (ELISA) (20).

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While the PRN test is generally accepted as the gold standard for mumps serology, it is cumbersome and time-consuming. As a result, it has been widely replaced by the faster and more convenient ELISA. Interestingly, however, numerous reports (25–34) indicate that the correlations between mumps PRN titers and IgG ELISAs, which use whole virus as the target antigen, range from poor (32) to significant but imperfect (31). Here, we report the development of mumps NP- and HN-specific ELISA methods that facilitate the analysis of sera with higher degrees of resolution than whole-virus ELISA methods. The NP protein was selected as an ELISA antigen because of its relative immunodominance and the HN protein because of its role in virus neutralization. Importantly, these data suggest that whole-virus ELISA may lead to an overestimate of the number of individuals that have HN-specific or neutralizing antibodies. The HN- and NP-specific ELISAs may be useful for future studies that attempt to identify a correlate of mumps protection. It is important to more fully understand the immune response to mumps to ensure that the vaccine(s) and vaccination strategies are as effective as possible.

MATERIALS AND METHODS

Human subjects and study design. Sera from the three separate cohorts described below were collected and tested after informed consent was obtained for all study participants and under the approval of the Centers for Disease Control Institutional Review Board (IRB).

Early revaccination cohort study. During 1990 to 1992, serum specimens were collected from 14- to 18-month-old infants before and after MMR vaccination as part of an IRB-approved study to address antibody persistence and assess the response to an early second dose of MMR. A subset of sera from 37 infants was chosen at random from this larger set for the purpose of this study. Sera were collected from infants at five time points, prevaccination (T_0), 2 to 3 months after first-dose MMR (T_1), 4 to 7 months after first-dose MMR (immediately prior to second-dose MMR) (T_2), 2 to 4 months after second-dose MMR (T_3), 2 years after first-dose MMR (T_4), and 4 years after first-dose MMR. The mean age of participants at the time of study enrollment was 15.1 months (range 13 to 31 months); 48.5% of the participants were female.

Third-dose MMR vaccination study participants. In a previously described study of antigen-specific B-cell responses following MMR vaccination (35), serum was collected immediately before and 3 weeks after the administration of a third dose of MMR vaccine. Only those individuals who had two previous doses of MMR (11 of 16 original volunteers) were chosen for this analysis.

Acute and convalescent paired sera. Acute and convalescent paired serum samples were collected from 45 suspected mumps cases as part of routine clinical diagnostic testing. Cases were considered confirmed based on the presence of either IgM in one or both serum samples or a ≥4-fold rise in PRN titer between the two serum specimens.

Cell and virus cultures. Vero cells (African green monkey kidney and epithelial cells; ATCC CCL-81) were maintained at 37°C in 5% CO_2 in DMEM supplemented with 5% fetal calf serum, penicillin, streptomycin, and L-glutamine. Mumps virus (Jeryl Lynn strain) was grown on Vero cells by seeding at a multiplicity of infection (MOI) of 0.01 and incubating at 37°C until the cytopathic effect was complete.

Plasmid construction and expression of recombinant proteins. Recombinant plasmids were constructed under approval of the CDC Institutional Biosafety Committee. The NP and HN genes from a stock of Jeryl Lynn vaccine virus (Merck) were amplified by reverse transcription (RT)-PCR using the following primers: NP forward (5′-CCGGAGCTCATGTCATCTGTGCTCAAGGCATTTG-3′), NP reverse (5′-GGCCTCGAGTTACTCATCCCAGTCTCCCAC-3′), HN forward (5′-CCGGAGCTCAAATGGAGCCCTCGAAACTATTTATAATG-3′), and HN reverse (5′-GGCCCCGGGTCAAGTGATGGTCAATCTGGC-3′).

PCR products were purified and cut with either SacI and XhoI (NP), or SacI and XmaI (HN). The inserts were then ligated into the plasmid pCAGGS (36), which was digested with the corresponding enzymes and pretreated with shrimp alkaline phosphatase (Roche). Sequence analysis indicated that both the NP and HN clones were derived from the strain of Jeryl Lynn that corresponds to GenBank accession number AF338106.

Vero cells were seeded at 400,000 cells per well of a 6-well culture plate 16 to 24 h prior to transfection. Plasmids were transfected using LT-1 transfection reagent (Mirus) according to the manufacturer's recommended protocol. Cells were harvested 48 h posttransfection with 300 μl/well of buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 1 mg/ml bovine serum albumin). One proteinase inhibitor cocktail tablet (catalog number 11836170001; Roche) was included per 10 ml of lysis buffer. Cells were scraped into lysis buffer and vortexed for approximately 30 s. Lysates were then spun at 16,000 × *g* in a microcentrifuge for 30 s to pellet cell debris. The supernatant was stored at −20°C. Expression of recombinant proteins was also verified by immunofluorescence microscopy (data not shown).

ELISA. ELISA testing was done by use of a commercially available method and an in-house method. The commercial test (mumps IgG ELISA II [catalog number 425900CE]; Wampole Laboratories, Inc.) was done as previously described (37). Briefly, the Wampole ELISA uses whole-mumps virus antigen (Enders strain). The manufacturer reports that the relative sensitivity of this test is 96.6% (95% confidence interval [CI], 94.0% to 99.3%), and the relative specificity is 90.4% (95% CI, 82.4% to 98.4%). According to the manufacturer's protocol, an index standard ratio (ISR) value is calculated for each specimen as follows. The manufacturer provides a calibrator sample and a correction factor (CF) that is specific for each lot of kit components. The average optical density (OD) (absorbance at 450 nm) of the calibrator sample is multiplied by the CF value to determine a cutoff OD (CO) value. The unknown specimen OD value is divided by the CO value to determine the ISR. The cutoff points for IgG antibodies to mumps virus, based on index standard ratio (ISR) values, were seronegative, ISR ≤0.90; indeterminate, ISR 0.91 to 1.09; and seropositive, ISR ≥1.10.

An in-house ELISA was developed using recombinant HN and NP proteins from the Jeryl Lynn strain of mumps virus. Recombinant mumps antigen lysate (described above) was titrated against known positive serum samples to determine the optimum concentration required for detection. The NP, HN, and untransfected Vero control antigens were diluted in phosphate-buffered saline (PBS) and used to coat ELISA plates (catalog number 439454; Nunc MaxiSorp) overnight at 4°C. Plates were washed 3 times with 300 μl PBS plus 0.1% Tween 20, and then were blocked for 30 min at 37°C in 100 μl of BLOTTO (PBS plus 0.1% Tween plus 5% dry milk). The block was replaced with 100 μl of fresh BLOTTO containing a 1:100 dilution of serum. Plates were incubated at 37°C for 1 h, then were washed 4 times with 300 μl PBS plus 0.1% Tween 20. Secondary detection antibody (horseradish peroxidase [HRP]-labeled goat α-human IgG [catalog number 474-1002]; KPL) was diluted 1:25,000 in BLOTTO and 100 μl was applied per well. Plates were incubated at 37°C for 1 h, then washed 4 times with 300 μl PBS plus 0.1% Tween 20. The colorimetric detection reagent was prepared immediately before use and consisted of 10 ml of 0.1 M citrate acetate (pH 5.5), 2 μl of 30% hydrogen peroxide, and 200 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) (5 mg/ml in dimethyl sulfoxide [DMSO]). We applied 75 μl of detection solution to each well and incubated the plates for 15 min. Then the reaction was stopped by the addition of 75 μl of 2 M phosphoric acid. Absorbance values were then measured at 450 nm. Background absorbance values measured from the untransfected Vero antigen control were subtracted from the absorbance measured from the NP and HN lysates. To normalize the antigen-specific ELISA measurements across experiments, we divided the background-corrected absorbance values of the test sera by the background-corrected absorbance values of a positive-control serum that was included with each experiment (38). Mumps NP-specific IgM was measured by ELISA using a method similar to that described for measles (39).

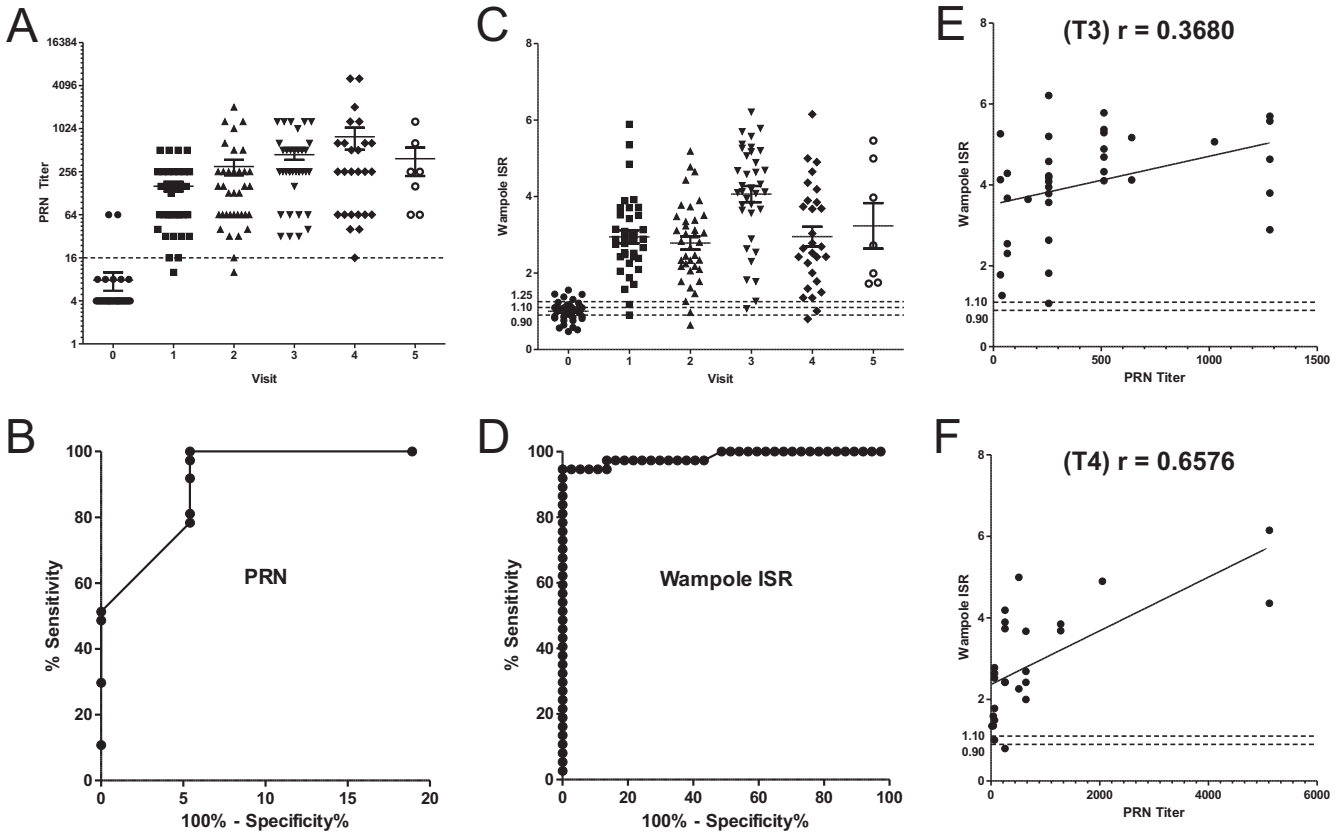


FIG 1 Plaque reduction neutralization (PRN) and commercial ELISA testing of infant sera pre- and postvaccination. Serum samples were collected from 37 infants at prevaccination (T_0), 2 to 3 months after first dose of MMR (T_1), 4 to 7 months after first MMR (T_2; also collected immediately before administration of second MMR), 2 to 4 months after second MMR (T_3), 2 years after first MMR (T_4), and 4 years after first MMR (T_5). Sera were tested by PRN (A), and ROC analysis was performed using the sera collected at the T_0 and T_3 time points as the negative controls and the test samples, respectively (B). Sera were tested by commercial ELISA (C) and ROC analysis of the commercial ELISA was performed using the T_0 and T_3 time points (D). PRN and commercial ELISA ISR values were compared and analyzed by linear-regression for the T_3 (E) and T_4 (F) time points.

Plaque reduction neutralization tests. The mumps vaccine strain Jeryl Lynn (JL5 large plaque variant and 3 times plaque purified) was used for plaque neutralization tests as follows. Virus was diluted to 1,000 PFU/ml in modified Eagle's medium (MEM) supplemented with Fungizone (Life Technologies), penicillin, streptomycin, gentamicin, L-glutamine, HEPES (pH 7.4), and 3% heat-inactivated fetal calf serum. Serum samples were heat inactivated at 56°C for 30 min. Heat-inactivated sera were serially diluted with MEM, then 60 μl of each serum dilution was combined with an equal volume of diluted virus (60 PFU). The serum and virus combination was incubated for 90 min at 37°C in 5% CO_2. Vero cells were seeded in 24-well plates and maintained in DMEM plus 5% fetal calf serum. Upon reaching confluence, the medium was aspirated from the cells and serum-plus-virus samples were added. Plates were incubated on a rocker table at 37°C in 5% CO_2 for 1 h. After the 1-h incubation, 1 ml of carboxymethyl cellulose (CMC) medium (1× Leibowitz medium, 2% carboxymethyl cellulose, 5% fetal calf serum, 0.1% sodium bicarbonate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml Fungizone, 250 μg/ml gentamicin, and 0.001% phenol red) was added to each well. Cultures were incubated at 37°C in 5% CO_2 for 5 days and then stained with crystal violet. The PRN end titer was determined as the highest dilution of serum that gave 50% or higher plaque reduction compared to the average number of plaques formed in the absence of serum.

RESULTS

Mumps IgG ELISA PRN titer pre- and post-MMR vaccination. Several reports have indicated that mumps-neutralizing antibody does not correlate particularly well with mumps IgG ELISA measurements (25–33). An explanation for these observations is that mumps antibodies predominantly measured by ELISAs which use whole-virus antigen differ from those measured by plaque neutralization tests. To test this hypothesis, we first examined these parameters using a well-defined set of serum specimens that were collected from infants prior to MMR vaccination and at multiple times following the first and second dose of MMR. We measured mumps PRN titers and mumps IgG levels using a commercial ELISA (whole-virus antigen; Wampole Laboratories, Inc.) and ELISAs specific for recombinant mumps nucleoprotein (NP) or hemagglutinin (HN) protein. The rationale for using this particular set of sera was to establish the cutoff values, sensitivity, and specificity for each test method and to determine the degree of correlation among the methods.

As shown in Fig. 1A, 35 of 37 prevaccination sera (T_0) had PRN titers of 1:8 or lower. Although 2 of the T_0 sera had PRN titers of 1:64, this could have been due to the presence of maternal antibody. All but one of the T_1 serum samples (collected at 2 to 4 months after the first dose of MMR) had PRN titers of 1:16 or greater. PRN titers remained essentially the same at T_2 (4 to 7 months postvaccination). The second dose of MMR was given immediately following the T_2 serum collection. Compared to the T_2 samples, the average PRN titer was slightly higher in the sam-

ples collected at T_3 (2 to 4 months following the second dose) and T_4 (~2 years after the first dose). Although fewer samples were available from T_5 (~4 years after the first dose), the mean T_5 PRN titer was similar to but slightly less than the earlier postvaccination time points. A receiver operating characteristic (ROC) curve analysis was done using the prevaccination and T_3 samples as the negative controls and test samples, respectively (Fig. 1B). ROC analysis indicated that the PRN test was 97.3% sensitive and 94.6% specific when 1:16 was used as the cutoff value. When 1:32 was used as the cutoff, the sensitivity was 91.9% and the specificity was 94.6%.

These same sera were tested for mumps IgG using a commercial ELISA (Wampole) according to the manufacturer's instructions (Fig. 1C). This kit uses whole mumps virus (Enders strain) as the antigen. The ELISA absorbance values (OD 450 nm) were converted to an index standard ratio (ISR) using a manufacturer-supplied lot-specific correction factor and a calibrator control measurement as described in the Materials and Methods. Although endpoint serum dilutions were not calculated, it was previously reported that the ISR values for the Wampole ELISA are linear across a wide dilution range (3). The results indicated that 14 of the T_0 sera had ISR values below the manufacturer's negative cutoff (<0.90), 10 sera had indeterminate values (between 0.90 and 1.10), and the remaining 13 T_0 had positive values (>1.10) but were near the cutoff. After the first dose of MMR, 36 of 37 sera at T_1 were positive, with ISR values >1.10. At T_2, 35 samples were positive, 1 was indeterminate, and 1 was negative. The average ISR value was noticeably higher after the second MMR at T_3, and 36 samples were positive and 1 was indeterminate. The average ISR value dropped at T_4 (2 years after the first MMR) down to a level that was comparable with the pre-second-dose level. ROC analysis of the Wampole ELISA using the T_0 and T_3 samples indicated a 64.9% specificity and 97.3% sensitivity at the manufacturer's positive cutoff (>1.10) (Fig. 1D). Although this was lower than expected, the ROC analysis indicated that the specificity could be improved to 86.49% by simply raising the cutoff to >1.25. The PRN titers and the commercial ELISA results from the T_3 and T_4 sera were compared as shown in Fig. 1E and F. There was essentially no correlation between the two parameters at T_3 (r = 0.3680). Although the correlation coefficient was higher at T_4 (r = 0.6576), it was of no predictive value.

Next, mumps NP- and HN-specific IgG levels were measured using recombinant antigens. Figure 2A and C show that prevaccination sera (T_0) gave very low absorbance values for both NP and HN. The signals appeared to peak by T_3, and ROC analysis was performed using the T_0 and T_3 data as before (Fig. 2B and D). The NP ELISA had 97.3% sensitivity and 97.3% specificity using a cutoff >0.360. The HN ELISA had 86.5% sensitivity and 97.3% specificity using a cutoff >0.306. The fraction of sera above the positive cutoff was higher for NP than for HN at each time point following vaccination. In addition, the response to HN as measured by ELISA appeared somewhat delayed relative to NP. There was also a substantial rise in the level of both NP and HN antibodies following the second dose of MMR (comparing T_2 to T_3). The T_3 absorbance values from the NP and HN ELISAs were compared to the T_3 PRN and Wampole ISR values (Fig. 3). There was essentially no correlation between the NP ELISA and PRN titer (r = 0.2175) (Fig. 3A) or the HN ELISA and Wampole ISR (r = 0.3812) (Fig. 3D). The correlation coefficients were higher but still weak for NP ELISA and Wampole ISR (r = 0.6731) (Fig. 3B) and for HN ELISA and PRN (r = 0.6183) (Fig. 3C).

Third-dose MMR vaccination in adults. To determine the effects of a third-dose of MMR on PRN titer, ISR, and antigen-specific IgG, we measured each of these parameters immediately prior to and 3 weeks after the administration of a third dose of MMR in 11 adult volunteers. One of the sera had a pre-third-dose titer that was just below the ISR positive cutoff, but the remaining sera were positive. All prevaccination sera had PRN titers of 1:40 or greater. As shown in Fig. 4A and C, the effects of a third MMR on PRN titer and HN IgG were none or modest. There were slight increases in the PRN titers and HN-absorbance for those samples with the lowest initial values, but the overall average was essentially unchanged. By comparison, there was a slight increase in the average commercial ELISA ISR and NP-specific ISR values (Fig. 4B and D).

Acute and convalescent paired sera. Acute and convalescent paired serum specimens were collected from 44 IgG seropositive individuals with suspect cases of mumps infection as part of routine clinical diagnostic testing. Each of these individuals was confirmed a mumps case either by the presence of mumps-specific IgM in one or both serum specimens or by a ≥4-fold rise in the mumps IgG PRN titer. We hypothesized that these individuals may have NP-specific antibodies but low PRN titers or low HN-specific antibody levels during the acute phase of infection.

Of these 44 individuals, 21 (47%) had ≥4-fold increases in PRN titers between acute and convalescent time points. It is possible that an increased PRN titer was not observed in certain serum pairs because the antibody had already boosted prior to the collection of the acute-phase specimen. The average times of acute-phase specimen collections were 5 and 3 days postonset for those with <4 and ≥4-fold rises in PRN titers, respectively. The average times of convalescent specimen collections were 22 and 16 days postonset (P = 0.0455). For further analysis, the individuals who had ≥4-fold increases in PRN titers and those who did not were considered separately. Of the 21 individuals who had a ≥4-fold rise in PRN titer, there was one individual who was IgM negative at both the acute and convalescent time points, and one who was IgM indeterminate at both times (data not shown). IgM was detected in one or both serum samples from the remaining 43 individuals.

As shown in Fig. 5A, there was a significant (P = 0.0041) difference in the average convalescent-phase PRN titers between the two groups but not between the average acute-phase PRN titers. Similarly, as shown in Fig. 5B and C, the two convalescent groups had significant differences in the Wampole IgG (whole-virus antigen) and HN-specific IgG ELISA measurements (P = 0.0088 and P = 0.0078, respectively). In contrast, there were no significant differences among the acute-specimen ELISA measurements or among any of the groups with respect to NP-specific ELISA measurements (Fig. 5D).

To determine the degrees of correlation between the PRN titers and each ELISA, all of the acute- and convalescent-phase sera were compared (Fig. 6). There were very poor correlations between PRN titers and both the Wampole ELISA and the NP ELISA (Fig. 6A and B). In comparison, low PRN titers were predictive of low HN ELISA measurements, but sera with high PRN titers gave variable HN ELISA results (Fig. 6C).

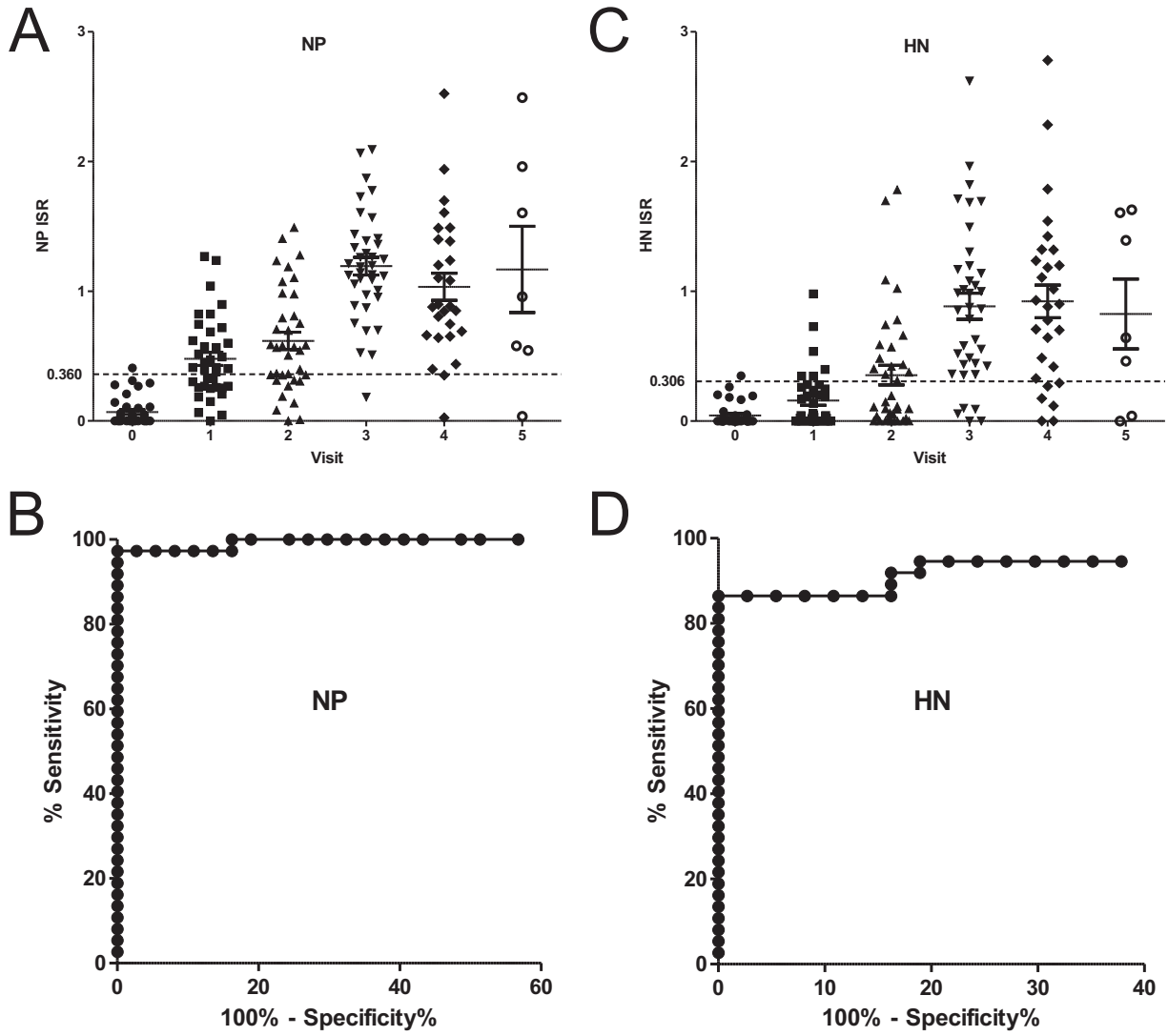


FIG 2 Mumps antigen-specific ELISA testing of infant sera. Infant serum samples collected pre- and post-MMR vaccination were examined by ELISA specific for the nucleoprotein (A and B) and hemagglutinin (C and D) proteins. ROC analysis was done using sera collected at the T_0 and T_3 time points.

DISCUSSION

It is not understood why some previously vaccinated (or wild-type infected) individuals are susceptible to mumps (re)infection since no specific correlates of protective immunity have been well defined. Although neutralizing antibody is thought to be essential for protection (40) and PRN titration is generally considered to be the gold standard for mumps serology, previous attempts to determine a reliable threshold for protective PRN titers have been unsuccessful (20). To add further ambiguity, there have been several reports of poor correlations between mumps PRN titration and various other serologic methods, such as ELISA and immunofluorescent assays (IFA) (25–34). These studies indicated that mumps serologic data must be interpreted with caution. Here, we hypothesized that the discordant results could be explained if ELISAs that utilize whole-virus as antigen predominantly detect nonneutralizing antibodies, such as those specific for the nucleoprotein.

We attempted to address these issues by developing ELISAs that are specific for the mumps NP and HN proteins, since ELISAs are more convenient to perform than PRN titration and are perhaps more sensitive and less subjective than immunofluorescent staining. We compared the antigen-specific ELISAs with a commercial ELISA (whole-virus antigen) and PRN titration by testing sera from three different cohorts, including (i) infant sera obtained before and after primary and early secondary MMR vaccination, (ii) adult sera obtained before and after a third MMR vaccination, and (iii) acute and convalescent paired sera following natural mumps infection. ROC analysis of each method using sera collected before and after vaccination indicated that each test had comparably high levels of sensitivity and specificity. The PRN and commercial ELISAs were somewhat more sensitive than the antigen-specific ELISAs (especially apparent at the T_1 and T_2 time points), presumably because they detect antibodies specific for other proteins in addition to NP and HN. The positive cutoff for the commercial test as determined by the manufacturer was slightly low according to these data. However, the false-positive

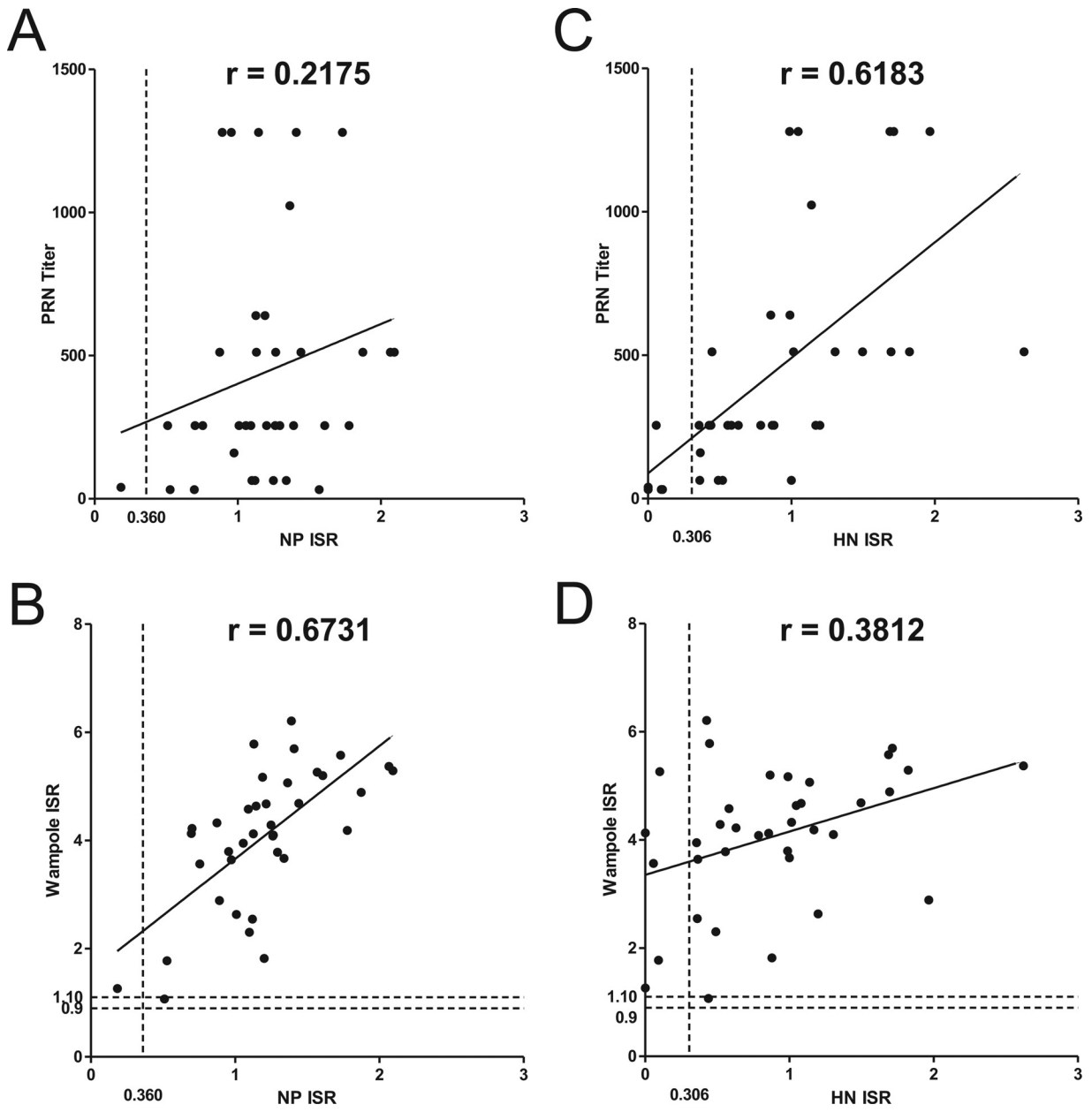


FIG 3 Correlation among PRN and ELISAs. NP- (A and B) and HN-specific (C and D) ELISA results from the infant sera collected at the T_3 time point were compared to PRN and commercial ELISA results from the same serum samples. The data were analyzed by linear regression and the correlation coefficient is indicated for each comparison.

results from the infant prevaccination sera were very near the cutoff, and these samples may have had some lingering maternal antibody since two of the sera had PRN titers of 1:64.

Similar to the reported findings mentioned above, our results indicate that the correlations between the commercially available ELISA and PRN titer were of little or no positive predictive value. Sera that had very low ISR values by the commercial ELISA generally had low levels of neutralizing antibody. However, sera that had moderate-to-high ISR values by the commercial ELISA had a wide range of plaque neutralization titers (Fig. 6). The NP-specific ELISA results also did not correlate with the PRN titers, but it did tend to correlate with the ISR values from the commercial ELISA. In contrast, the HN-specific ELISA results did not correlate with the commercial ELISA results but tended to correlate best with the PRN titers.

These results partially confirm and extend data recently reported by Matsubara et al. (32). Using an immunofluorescence method to detect serum antibody specific for the nucleoprotein (NP) and hemagglutinin (HN) proteins, they reported that whole-virus ELISA may predominantly detect NP-specific antibody (32). Immunofluorescence, however, is also cumbersome to perform on a large scale and it can be subjective, particularly when used to detect very low concentrations of antibody. This could explain the surprising negative correlation reported between HN antibody

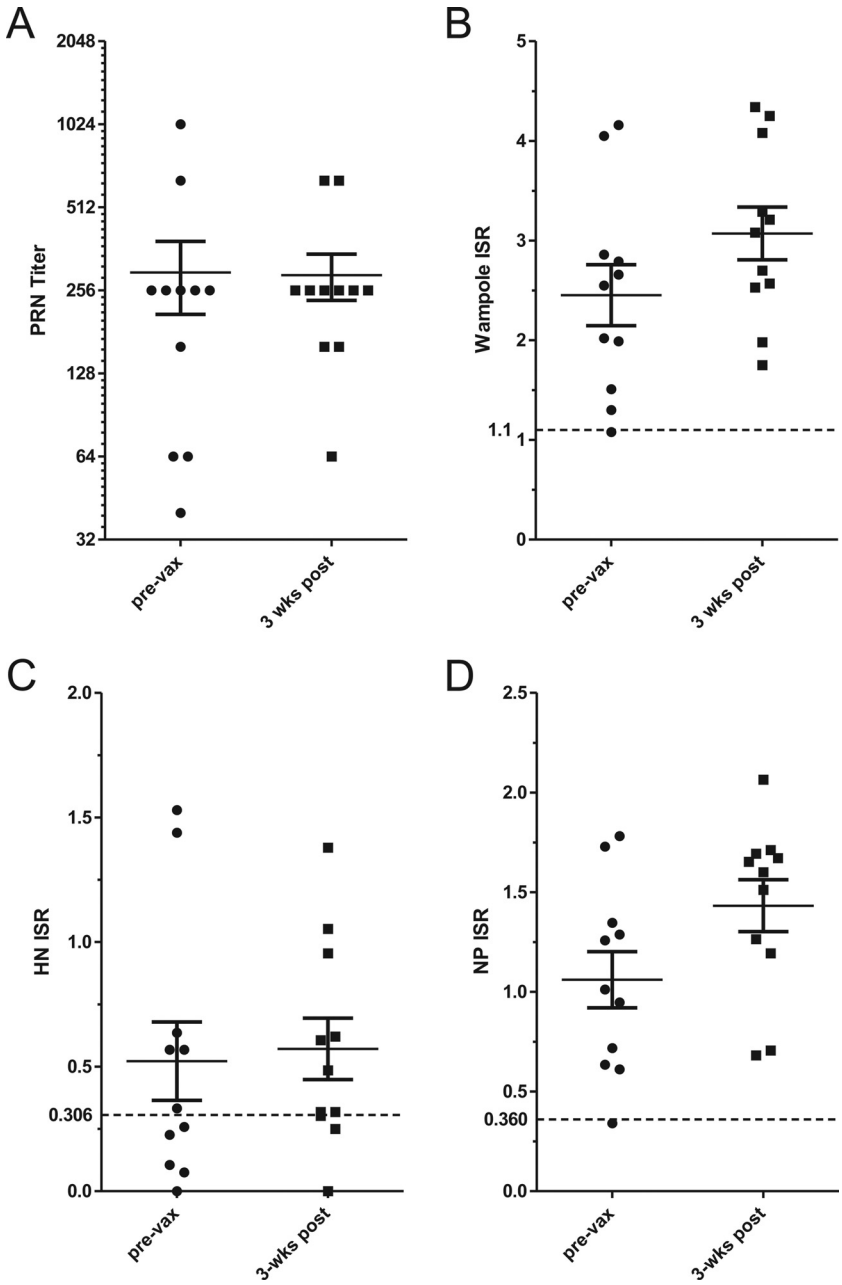


FIG 4 Serologic testing of serum samples collected before and after administration of a third dose of MMR. Sera were collected from 11 volunteers who had previously received two doses of MMR, immediately prior to receiving a third dose of MMR. Sera were then collected at 3 weeks postvaccination and tested by PRN (A), commercial ELISA (B), HN-specific ELISA (C), and NP-specific ELISA (D).

and PRN titer, since HN is considered to be a neutralizing target and NP is not (41). Regardless, their data also indicate that neither whole-virus ELISA nor HN-specific immunofluorescent methods are especially useful for identifying samples that have low levels of HN-specific antibody.

Although none of the correlations among the different tests reported here were strong enough to justify substitution of one method for another, the data suggest some points that may be important to consider moving forward. First, the NP protein seems to be the dominant antibody target compared to HN. This was most recently reported by Matsubara et al.(32) and also previously reported by Linde et al. (34), whose results further indicated that detection of NP IgG occurs approximately 2 weeks prior to HN IgG during the course of natural infection. This suggests that the antibody responses to NP and HN may be fundamentally different in some way. Here, we note that the majority of individuals who were seropositive by the commercial ELISA appeared to have relatively high levels of NP-specific antibodies but with wide variations in PRN titers and HN-specific ELISA absorbance levels. This result contrasts with results for measles serology, which has better correlation between PRN and whole-virus ELISA and for which PRN is considered the most sensitive testing method

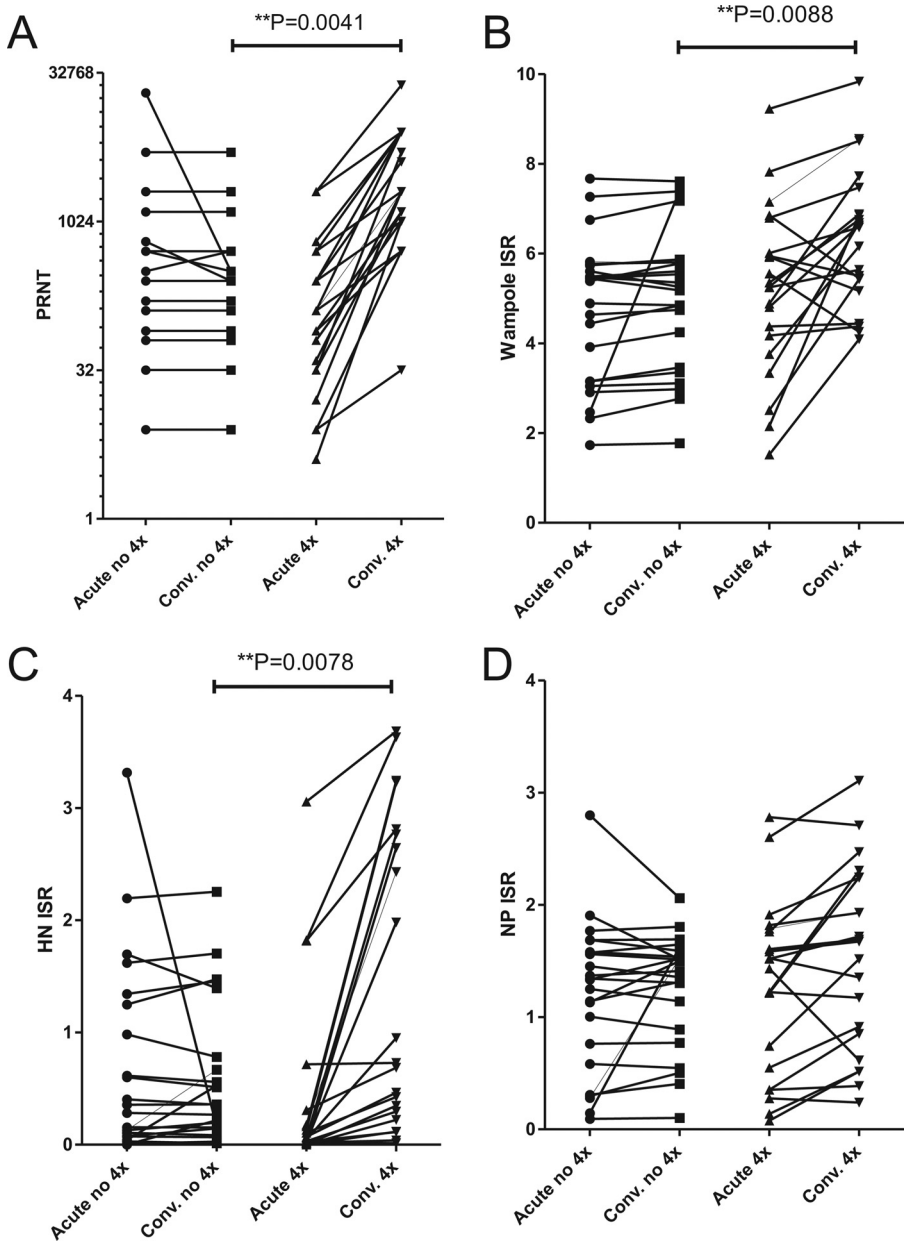
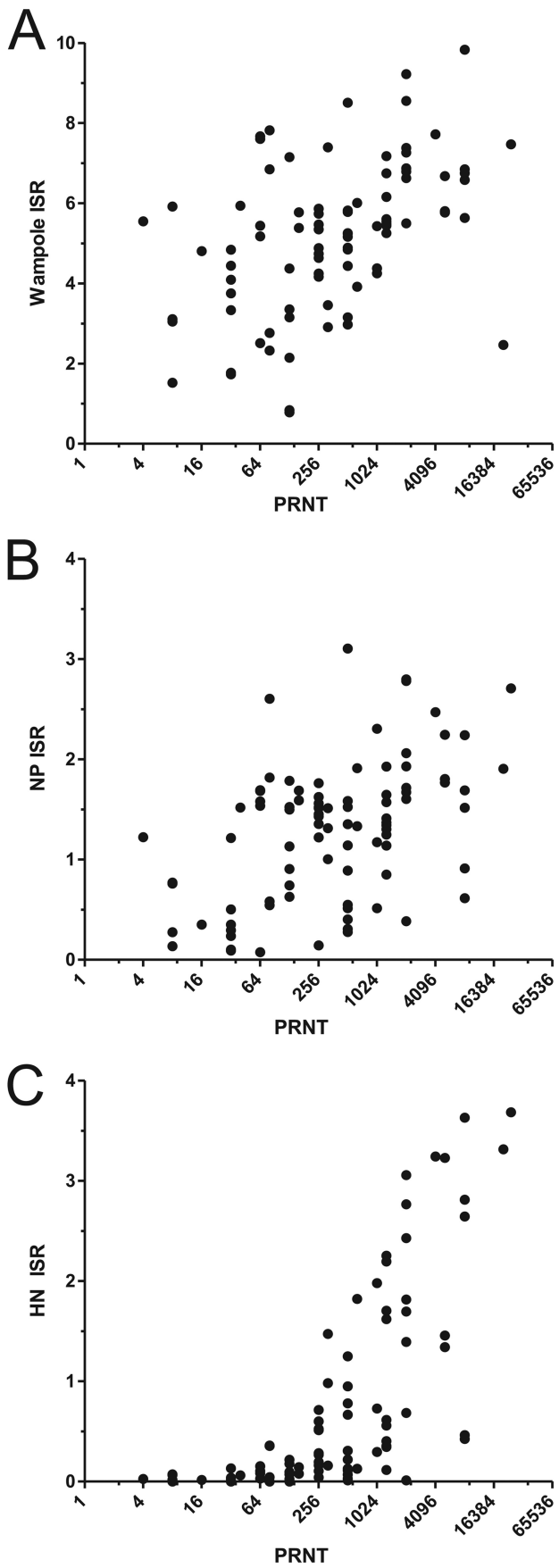


FIG 5 Serologic testing of acute- and convalescent-phase sera from confirmed mumps infections. Acute/convalescent paired sera from confirmed mumps cases were tested by *in vitro* plaque reduction neutralization (A), commercial ELISA (B), HN-specific ELISA (C), and NP-specific ELISA (D).

(42–46). In addition, measles serology benefits from having an international reference standard with a level of neutralizing activity that correlates with protection (47).

It is possible that the detection of NP-specific antibody by ELISA is more efficient than detection of HN-specific antibody. Paramyxoviruses such as mumps produce more proteins from the genes that are located at the 3′ ends of the genome (such as NP) relative to those that are close to the 5′ ends (HN). Because whole virus is used as the antigen in the commercial ELISA, there is likely more NP protein than HN available for detection. Also, since NP is a globular protein and HN is membrane bound with multiple glycosylations, it is possible that antibody recognition epitopes are not equally well represented on each protein in the ELISA. It may be more desirable to perform the HN ELISA in a capture format to optimize the availability of epitopes for antibody binding. Furthermore, it is possible that additional epitopes are available only when HN is coexpressed with other viral proteins, such as the fusion protein (F). Although we successfully expressed recombinant mumps F protein and tested it by ELISA, the absorbance values were too low to be useful (data not shown). Coexpression of recombinant F and HN proteins may be a way to improve the correlations between the PRN test and ELISA. Taking these things into consideration, we were able to detect high levels of antibody in many sera with the HN-specific ELISA. At a minimum, this suggests that not all HN epitopes were limiting in the assay. In addition, the potential shortcomings of the HN-specific ELISA do



not explain the extreme variability in PRN relative to ISR values or NP antibody detection. One of the most important conclusions from these data is that care must be taken when interpreting ELISA results, especially those based on whole-virus antigen. While it may be reasonable to assume that an individual who is seronegative by ELISA likely does not have neutralizing antibody, no assumptions can be made about the levels of neutralizing antibodies in individuals who are seropositive by ELISA. Measurements of seroconversion that mostly detect NP antibody without taking into account the level of neutralizing antibody may overestimate the level of protection.

A second consideration that these data point to is mumps antibody boosting. As might be expected, we observed rises in PRN titers and ELISA antibody measurements following the second dose of MMR (compare T_2 and T_3 in Fig. 1A and C and Fig. 2A and C), but the effects were transient and the levels of antibodies appeared to return to near baseline by 2 years following the second dose. From these data alone, the precise benefit of a second MMR with regard to mumps protection is not clear. Although the Advisory Committee on Immunization Practices (ACIP) initially recommended a routine second-dose of MMR mainly to address measles primary vaccine failure (48), routine two-dose MMR vaccination had the added benefit of a further reduction in mumps disease incidence (1). In light of this, a second dose of MMR may be important for mumps protection, especially when considering reports of the waning of mumps-neutralizing antibody (14, 31, 49). The waning of mumps-neutralizing antibody is concerning because mumps PRN titers tend to be low even after recent vaccination or wild-type infection (18, 24, 50–53). Since, as mentioned, genetically divergent strains can be neutralized with only slight variations in serum titers (13–15), it has been suggested that individuals with extremely low PRN titers may not be protected against some wild-type strains (15). Furthermore, it is not clear how long individuals who have been infected with wild-type virus retain a protective level of immunity in the absence of additional natural boosting. Also, the population of individuals who have been vaccinated but not naturally infected also continues to grow. Therefore, the level of herd immunity may not be as robust as it might be if wild-type virus were endemically circulating and providing regular asymptomatic boosting. Although we examined only a small number of individuals following administration of a third dose of MMR, there was very little effect on the average PRN titer or HN-antibody level in this cohort. Increases in PRN titer and HN antibody were observed in those individuals who had the lowest initial titers, and there was a slight overall increase in ISR values and NP antibodies, but the effect was modest.

Examination of acute and convalescent paired sera from confirmed cases of breakthrough mumps infection among vaccinees revealed that some individuals had >4-fold rises in PRN titers (Fig. 5A). These sera also had a corresponding rises in HN-specific antibodies (Fig. 5C), but not necessarily rises in NP-specific antibodies (Fig. 5D). While these data suggest that increases in HN antibody may raise the neutralization titer, there were several individuals who had relatively high PRN titers and HN antibody

FIG 6 Correlations between PRN titers and ELISA ISRs. Plaque reduction neutralization titers of all acute and convalescent specimens are compared with titers measured by a commercial ELISA (A), NP-specific ELISA (B), and HN-specific ELISA (C).

levels during the acute phase of infection, yet they were still obviously susceptible. Since the majority of the acute-phase samples had PRN titers ≥1:16, it is possible that the antibody had already boosted to a certain degree by the time the acute-phase samples were collected. It is also possible that not enough time had elapsed between the collections of the acute- and convalescent-phase samples to allow for further increases in antibody titers. Alternatively, this may also indicate that PRN titer and HN antibody levels are not necessarily reliable measures of protection.

From these limited data, it is also not clear if or when a third dose of vaccine would be of added benefit to the majority of individuals. A third dose may boost the levels of antibodies in those with low titers, but again it is not known what if any level of neutralizing antibody may be protective (54). If the dominant antibody response is to NP rather than HN, an additional dose of vaccine may simply skew the response further. Nonetheless, if subclinical boosting due to naturally circulating wild-type mumps had an important role in keeping the incidence of disease low, it may be reasonable to provide a third dose of MMR as an outbreak control measure. In fact, during two recent outbreaks, a third dose of MMR was given as a control measure and there was a decline in mumps incidence shortly afterward (55–57). In both outbreaks, however, it was not clear if the reduction in mumps incidence was a direct result of the third-dose intervention or if the timing of the intervention simply coincided with what would have otherwise been a natural decline in incidence. Furthermore, it was noted that the frequency of reported complications, such as orchitis, deafness, meningitis, and oophoritis, was lower among symptomatically infected two-dose vaccinees than among individuals who received fewer doses or who had an unknown vaccination history (19). This indicates that while vaccination may not provide complete protection in all circumstances, it does provide some important measures of protection that should not be disregarded.

A better understanding of which immunologic parameters best correlate with protection will be necessary to make scientifically grounded improvements to the mumps vaccine or vaccination strategy. However, it may be naive to assume that there are any specific parameters that will reliably ensure protection, since the conditions of exposure can be so variable. For example, recent mumps outbreaks have occurred predominantly in environments such as college dormitories, summer camps, and boarding schools that facilitate high frequencies and intensities of exposure (1, 2). Such conditions may overwhelm the immune systems of individuals who have low levels of immunity that may be protective under less intense circumstances (19, 24). Furthermore, the nature of the immune response to mumps seems to be inherently weak by some measures. Besides inducing typically low levels of neutralizing antibody, multiple reports indicate that the frequency of mumps-specific memory B-cells is also very low (35, 58, 59). It may be reasonable to try to improve the neutralizing antibody response by (re)directing it toward HN rather than NP, but other effects of antibodies, such as complement fixation, should not be overlooked (60, 61). Also, antibodies that are nonneutralizing *in vitro* may have other underestimated roles *in vivo* (62). Future attempts to define correlates of mumps protection will likely need to examine the immune response in much greater detail and specificity than afforded by whole-virus ELISA. In addition, careful characterization of mumps-specific immunity in close contacts of confirmed mumps cases will be essential.

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