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Evaluation of Enzyme-Linked Immunosorbent Assay (ELISA) for Mumps Virus Antibodies (40451)

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The determination of serum neutralizing antibodies (NT) has been the most sensitive and specific method for detecting past or recent exposure to mumps virus (1–3). The presence of nonspecific inhibitors makes hemagglutination inhibition (HI) antibody assays less sensitive and cross reactive antibodies to other paramyxovirus infections may cause false positive readings when complement fixation (CF) is used (4, 5).

Recently, four new tests have been described and advocated for serological studies of mumps antibodies. Vaananen *et al.* described a hemolysis-in-gel technique (HIG) which they found to be more sensitive than HI in detecting postvaccination antibodies due to killed vaccine (6). Norrby and his coworkers recently described a hemolysis inhibition (HLI), a single radial immunodiffusion (SRID) and a mixed hemadsorption technique for serodiagnosis of mumps virus infection and evaluation of mumps immunity (7). SRID and HLI seemed useful for serodiagnosis while mixed hemadsorption, although technically rather complicated, was advocated for determining immunity.

Recently several enzyme-linked immunosorbent assays (ELISA) have been developed for the determination of virus antibody levels (8, 9). These ELISA techniques have proven sensitive, specific and simple to perform (10). We report here the comparison of an ELISA mumps test with NT, HI, CF and HIG tests.

Materials and methods. Serum samples. Pre- and postvaccination sera from 15 individuals vaccinated with Jeryl-Lynn vaccine were available (11). The postvaccination samples had been collected about one month after the administration of the vaccine. In addition, 10 sera from six individuals collected 1.5 to 8 years after the vaccination were studied. Also pools of positive and negative sera used in the neutralization tests as standards were included in the series.

The neutralizing mumps virus antibody levels had been determined earlier (3). All sera were coded before they were sent to NIH. Prior to testing, they were stored at −20°.

Antigens. Mumps virus antigen (prepared by Dr. G. Castellano, Bethesda, MD) was harvested from allantoic fluid of chick embryos and was used in the HI, CF and ELISA tests. It had a HI titer of 500. A control antigen of uninfected allantoic fluid was obtained from the same source.

ELISA for mumps antibodies. The principle and the technical performance of ELISA test for virus antibodies have been described (12). Briefly, antigen diluted into phosphate-buffered saline (PBS, pH 7.4) was incubated overnight at +4° in disposable polystyrene cuvettes (Finnpipette-Labsystems, Helsinki, Finland). In the morning, the cuvettes were washed with PBS supplemented with 0.05% Tween 20 (PBS-Tween). Serial dilutions (half logarithm steps) of the reference serum and three dilutions (1/50, 1/500 and 1/5000) of serum samples were made in "filler" solution (PBS + 1% bovine serum albumin + 0.05% EDTA) and then incubated in the sensitized cuvettes for 90 min at +37°. After washing with PBS-Tween, heavy-chain-specific anti-human IgG (Orion Diagnostica, Mankkaa, Finland) conjugated with alakline phosphatase was incubated for 60 min at +37°. The immunosorbent purification and conjugation procedures have been described earlier (12). The conjugate was diluted into "filler" used also for serum dilutions. After incubation the excess conjugate was washed out with PBS-Tween and substrate (PNPP) added for 30 min at +37°. The reaction was then stopped by adding 1.5 *M* NaOH. The intensity of the yellow color was measured by using FP-9 photometer (Finnpipette-Labsystems, Helsinki, Finland) which measures the optical densities through the bottoms of the cuvettes

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via nine optical channels (10). The photometer is equipped with a programmable calculator. The amounts of antibody were expressed as logarithmic values which were obtained by comparing the test results with a standard curve made from a reference serum. A zero-point was determined by analyzing negative sera. This method of quantitation is analogous to our previous "ED-method" except that the scale is now positive: the higher the logarithmic value the higher the antibody activity of the test sample. A value of 0 indicates that no antibodies could be found. A change from 1.0 to 2.0 indicates a tenfold increase. A change of 0.2 was regarded as significant in the test (10).

Hemoloysis-in-gel (HIG). Commercially available HIG plates were used ("Orivir", Orion Diagnostica, Mankkaa, Finland). They had been prepared by mixing cells sensitized with mumps virus antigen with agar and complement (6). In the presence of antibodies, antigen–antibody complexes form on the surface of red cells which are then lysed by the complement. Five microliter of 1:2 diluted serum was applied into each well, the plates were sealed and incubated at +37° for 16 hr. The diameter of the clear hemolytic zone was then measured. The radius of the hemolytic zone is directly proportional to the antibody activity in the serum.

Conventional serological tests. Microtiter CF, as previously described (13) was used to determine the levels of CF antibodies. Two full units of complement and four units of antigen was used. HI antibody levels were determined by using chicken red cells and four units (HA) of the antigen. Prior to HI testing the sera were absorbed with Kaolin and red blood cells to remove nonspecific inhibitors (3). The NT test has been described earlier (3).

Results. The overall correlation between the tests was found to be quite satisfactory although differences were found both in the sensitivity and specificity of the tests (Tables I and II). ELISA was the most sensitive in detecting significant increases in antibody levels following vaccination: in 14 of 15 cases a significant rise was seen, while in only one case no such increase was found (Case No. 8). This particular individual had rather high initial titers which probably protected from the vaccine virus. By NT an increase in serum antibodies was seen in 13 of 15 cases but in one the increase was only twofold and not regarded significant. CF gave a significant (fourfold or greater) increase in 11 cases, in three further cases a twofold increase was seen. Interestingly in one case (No. 12) CF failed to detect antibodies in the prevaccination serum and gave only a very weak reac-

TABLE I. ANTIBODY LEVELS IN SERA OF 15 PERSONS VACCINATED WITH LIVE MUMPS VACCINE AS DETERMINED BY ELISA, HEMOLYSIS-IN-GEL (HIG), SERUM NEUTRALIZATION (NT), HEMAGGLUTINATION INHIBITION (HI) AND COMPLEMENT FIXATION (CF) TECHNIQUES.

	ELISA		HIG		NT		HI		CF	
Case No.	Pre*	Post*	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	0.0	1.2	0	5	<2	16	<4	4	tr***	16
2	0.0	1.2	0	5	<2	16	<4	8	tr	32
3	0.0	1.0	0	3	<2	32	<4	4	tr	16
4	0.0	1.4	0	0	<2	8	<4	4	tr	8
5	0.5	1.1	4	4	8	8	4	4	tr	4
6	0.0	1.3	0	6	<2	4	<4	4	<4	8
7	0.0	1.9	0	6	<2	16	<4	32	tr	32
8	1.3	1.1	4	4	≥8	8	4	4	tr	4
9	0.0	1.6	0	6	<2	64	<4	32	<4	64
10	0.0	1.3	0	6	<4	8	<4	4	<4	16
11	0.0	1.2	4	6	<2	8	<4	4	tr	32
12	0.3	1.0	0	6	≥8	32	4	16	<4	tr
13	0.0	1.4	3	4	<2	16	<4	4	tr	16
14	0.6	1.1	5	5	≥8	16	4	4	8	16
15	0.0	1.2	4	5	<4	32	<4	8	<4	16

* Pre = Prevaccination sample; post = serum sample taken 1 month after the vaccination.
** Diameter of the hemolyzed zone in mm.
*** tr indicates a weaker than 4+, but a definite complement fixation in 1:4 dilution.

TABLE II. ANTIBODY LEVELS IN LATE POSTVACCINATION SERA AS DETERMINED BY ELISA, SERUM NEUTRALIZATION (NT), HEMAGGLUTINATION INHIBITION (HI) AND COMPLEMENT FIXATION (CF) TECHNIQUES.

Case No.	Time after vaccination	Method			
		ELISA	NT	HI	CF
16	8 years	0.1	1	<4	8
17	1.5 years	1.4	1	<4	c+*
	2 years	1.4	1	<4	"
18	1.5 years	0.9	1	<4	16
	2 years	0.8	1	<4	16
19	4 years	1.0	1	<4	16
20	3 years	0.2	1	<4	tr**
21	1.5 years	0.6	1	4	8
	2 years	0.5	1	4	16
	5 years	0.2	1	4	16

* Positive with control antigen.
** See note Table I.

tion in the postvaccination sample although NT, ELISA and HI all indicated the presence of antibodies in both samples and also HIG in the second sample.

HIG and HI were less sensitive in detecting significant increases in the vaccinees. In only five cases a significant increase in HI antibodies was found. HIG was more sensitive, it detected a significant increase in eight cases. The lower sensitivity of HI compared to other methods was also obvious when the late postvaccination sera were studied (Table II). HI detected antibodies in only 3/10 sera while both ELISA and NT indicated the presence of antibodies in all samples. The late postvaccination sera were not studied by HIG.

A comparison of the specificity of the techniques was made by comparing the results with those obtained with NT. The best specificity was found in ELISA which in no instance gave a discrepant positive or negative result (Table III). Also HI was very specific: it gave no false positive reactions although, because of its lesser sensitivity, a false negative reading was found in the seven samples with low NT antibodies. HIG and CF showed somewhat lesser specificity with both false positive and false negative readings. In three prevaccination sera a hemolysis zone was clearly visible in spite of the absence of mumps antibodies as indicated by NT, ELISA and HI. Interestingly, the same three samples also showed slight reactivity in CF. In one postvaccination serum no HIG antibodies could be detected. CF found a distinct although weak positive reaction (marked as "tr" in the tables) in seven prevaccination sera where antibodies were not present according to other methods. In case No. 12 CF showed no antibodies in the prevaccination serum and only low antibody levels ("tr") after the vaccination although other tests indicated the presence of antibodies in the first sample and a significant rise in the second. One sample with activity towards control antigen, showed a clear positive reaction in CF.

Discussion. Previous serological studies have shown that the determination of serum neutralizing antibody levels against mumps virus would be the best method to determine the immunity of an individual (5). HI, although specific, is not sensitive enough while crossreactive antibodies due to related viruses make CF less useful. In this study, a complete agreement was observed between the results of NT and ELISA. In fact, ELISA was somewhat more sensitive since it detected a significant increase in antibody levels in two cases where NT indicated only a twofold increase or no increase at all.

Several technical features make ELISA more practical for immunity studies than NT. ELISA is very simple to perform, commercial

TABLE III. COMPARISON OF NT TO THE SENSITIVITY AND SPECIFICITY OF OTHER SEROLOGICAL METHODS IN DETECTING MUMPS VIRUS ANTIBODIES.

Category	NT	ELISA	HIG	HI	CF
No. of significant increases (total 15 patients)	12	14	8	5	11
No. of false positive results:					
-pre-vaccination sera (15)*		0	3	0	7
early-post-vaccination sera (15)		0	0	0	0
late-post-vaccination sera (10)		0	ND	0	0
No. of false negative results:					
-pre-vaccinations sera (15)		0	0	0	1
early-post-vaccination sera (15)		0	1	0	0
late-post-vaccination sera (10)		0	ND	7	0

* Number of patients.

reagents are readily available and the performance can easily be automated for large scale screening studies. No pretreatment of the serum samples is needed and very small amounts of sera are enough to complete the test. We have used 50 μl capillaries to collect fingerprick samples from clinic patients and used the sera successfully in antibody assays in ELISA (unpublished). A good general standardization of the test is also achievable: We have previously shown that by first determining the actual amount of specific antibodies in a positive test standard, the amount of antibodies can be expressed directly in microgram/ml of serum (12). A generally available standard with known amounts of mumps antibodies in micrograms/ml would make the standardization of the test in different laboratories very simple.

The use of three tenfold dilutions of the serum samples usually helps in the evaluation and precise quantitation of the results. However, when screening studies are conducted for the detection of susceptible individuals, a simpler laboratory scheme can be used, i.e. one single dilution (1/50) in duplicate tested with mumps and control antigens. Whatever the modification, the use of reference serum and a standard curve is important for evaluation of the acceptable levels of background and to distinguish between low positives and negatives. In our experience 1/50 dilution conveniently eliminates nonspecific background and allows the determination of the zero-point simply from the optical reading which corresponds the leveling off of the standard curve. 1/50 dilution also permits an exact quantitation in a large majority of adult and adolescent sera. Only when the amount of mumps antibodies in the serum is so high that the optical reading falls into the upper flat area of the standard curve are additional dilutions required for exact quantitation of mumps virus antibodies.

HIG has been shown to be somewhat more sensitive than HI in detecting immunity against mumps (6). The simple technical performance of the test makes it a tempting alternative for screening purposes. When commercially available plates are used, the only steps to be performed by the laboratory are the application of the serum samples and the measuring of the clear zone. However, occasional false negative and false positive readings were observed. HIG might therefore be less desirable for screening studies although for the serological diagnosis of mumps in the clinical virus laboratories it would complement the conventional methods.

Is there a need for a good and sensitive test for mumps susceptibility since live Jeryl-Lynn vaccine seems to give a long lasting protection against mumps infection (14)? Mumps antibody levels seemed to decline in some of the cases and the fact that low levels did not seem to be protective against the vaccine indicates that the protective antibody levels might eventually be lost. If concurrent with this underprotected population, a substantial proportion of children remain unvaccinated an epidemic affecting both children and adults may occur. By using a good and readily applicable screening method for the susceptibility of the population, vaccination programs can be more effectively planned and promoted. A similar ELISA has been applied to studies of measles, rebella and vaccinia antibodies and the occurrence of these antibodies probably also reflect the efficacy of vaccination programs. ELISA would offer a useful large scale screening method to monitor various aspects of vaccination induced immunity in the general population.

Interestingly, the presence of low levels of antibodies in the prevaccination sera of some individuals did not prevent them from responding to the live vaccine. This would indicate that the presence of low levels of antibodies might not be protective. If antibody levels alone would determine the susceptibility of an individual to mumps virus infection, three of the cases whose late postvaccination sera were studied in the present series had antibody levels similar to those observed in these prevaccination sera and would thus be susceptible to reinfection. Their NT antibody titers were equal to one and, in fact, in an earlier seroepidemiological study where reinfections did occur, they were exclusively seen in individuals whose NT was ≤ 1. Thus, while in seroepidemiological screening studies the absence of mumps antibodies by using ELISA would be a useful indicator of the

prevalence of susceptible individuals in the population, in an individual case the detection of low levels of antibodies may not rule out the possibility of reinfection.

Summary. Pre- and postvaccination sera from individuals who had been vaccinated with live Jeryl-Lynn vaccine were assayed for mumps antibodies by using serum neutralization (NT), enzymed-linked immunosorbent assay (ELISA), hemolysis-in gel (HIG), hemagglutination inhibition (HI) and complement fixation (CF) techniques. ELISA was found to be equally specific and somewhat more sensitive than NT. HIG was less sensitive than either of these two and, with some specimens, less specific. However, it was more sensitive than HI. CF gave several low positive readings which were interpreted as false positive since they were found in prevaccination sera and other tests failed to detect antibodies in these samples. The ELISA mumps test can be used in place of the more expensive and time consuming neutralization test for screening epidemiology, documenting immunity and establishing the serological diagnosis.

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1. Bashe, W. J., Jr., Gotlieb, T., Henle, G., and Henle, W.: J. Immunol. **71,** 76 (1953).
2. Weibel, R. E., Stokes, J., Jr., Buynak, E. B., Whitman, J. E., Jr., and Hilleman, M. R., N. Engl. J. Med. **276,** 245 (1967).
3. Buynak, E. B., Whitman, J. E., Jr., Roehm, R. R., Morton, D. H., Lampson, G. P., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **125,** 1068 (1967).
4. Cook, M. K., Andrews, B. E., Fox, H. H., Turner, H. C., James, W. D., and Chanock, R. M., Amer. J. Hyg. **69,** 250 (1959).
5. Henle, W., *in* "Diagnostic Procedures to Viral and Ricketssial Infections" (Lennette and N. J. Schmidt, eds.) 4th ed. p. 457. Amer. Public Health Ass. New York (1969).
6. Väänänen, P., Hovi, T., Helle, E.-P., and Penttinen, K., Arch. Virol. **52,** 91 (1976).
7. Norrby, E., Grandien, M., and Örvell, C., J. Clin. Microbiol. **5,** 346 (1977).
8. Voller, A., and Bidwell, D. E., Brit. J. Exp. Pathol. **57,** 243 (1976).
9. Leinikki, P. O., and Pässilä, S., J. Clin. Pathol. **29,** 1116 (1976).
10. Leinikki, P. O., and Pässilä, S., J. Infect. Dis. **136,** s294 (1977).
11. Davidson, W. L., Buynak, E. B., Leagus, M. B., Whitman, J. E., Jr., and Hilleman, M. R., J. Amer. Med. Assoc. **201,** 995 (1967).
12. Leinikki, P. O., Shekarchi, I., Dorsett, P., and Sever, J. L., J. Clin. Microbiol. **8,** 419 (1978).
13. Sever, J. L., J. Immunol. **88,** 320 (1962).
14. Weibel, R. E., Buynak, E. B., McLean, A. A., and Hilleman, M. R., Pediatrics **56,** 380 (1975).

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