

Measurement of Parainfluenza-3 Virus Antibody by the Single Radial Hemolysis Technique

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A single radial hemolysis assay for parainfluenza-3 virus antibody is described as a simple, sensitive, accurate, and precise alternative to hemagglutination inhibition and serum neutralization tests. A highly significant correlation exists between single radial hemolysis zone areas and hemagglutination inhibition titers of both serum and nasal secretion samples. Antibody conversions equivalent to as little as a twofold rise in hemagglutination inhibition titer are readily and reliably detected in sera and nasal secretions. Single radial hemolysis and hemagglutination inhibition activity was demonstrable in the immunoglobulin G fraction of a hyperimmune parainfluenza-3 virus antiserum.

Initial serological studies with bovine parainfluenza-3 (Pi-3) virus utilized complement fixation, serum neutralization (SN), and hemagglutination inhibition (HI) to measure virus antibody (1, 5-7). There was good agreement between the results of these tests but HI was found to be the most sensitive and has since been widely employed. However, greater sensitivity of SN over HI has been described in a microtiter system (12).

In both SN and HI assays, a twofold sample dilution series is usually employed and the minimum accepted significant difference between antibody titers is fourfold. The accuracy and resolution of these assays is thus limited by the size of the dilution step used. It is also difficult to achieve reproducible results from test to test with HI, and SN suffers from the disadvantage of being a relatively complex and lengthy assay procedure.

A single radial hemolysis (SRH) method for measurement of influenza virus antibody has recently been described (13), and this technique has been extended and adapted in the current work to provide a simple, sensitive, and precise alternative assay for Pi-3 virus antibody.

MATERIALS AND METHODS

Viruses. Bovine Pi-3 virus was grown in primary calf kidney cell cultures. Two isolates were used. (i) "Compton" virus was isolated at the Institute for Research on Animal Diseases, Compton, Berks, England, in 1968 from an uninoculated calf kidney cell culture and fully characterized as Pi-3 virus (Janice C. Collins, Ph.D. thesis, Univ. of Reading, Reading, 1971). It has since undergone multiple passages in

calf kidney cell cultures and has a strong hemagglutinin activity, routinely reaching titers of 512 to 1024 hemagglutinating units/0.025 ml of tissue culture fluid. (ii) J121 virus was isolated from a naturally occurring case of calf respiratory disease (2) and has relatively weak hemagglutinin activity, routinely reaching titers of only 16 to 32 hemagglutinating units/0.025 ml of culture fluid. The infectivity titers of the fluids are, however, high at 1×10^9 to 5×10^9 plaque-forming units/ml.

Sera and nasal secretions. These were kindly provided by E. J. Stott and were derived from field and experimental calves sampled in the course of investigation of Pi-3 virus infection. Nasal secretions were collected by the method of McKercher (10). All sera and nasal secretions were inactivated at 56 C for 30 min before use.

Erythrocytes. Adult bovine erythrocytes were collected in Alsevers solution and stored at 4 C for up to 1 week. They were washed three times in phosphate-buffered saline before use.

SRH technique. The method followed was essentially that described by Russell et al. (13). The erythrocyte concentration in the gels and sample diffusion time were satisfactorily standardized on the basis of their results.

Equal volumes of packed erythrocytes and Analar sodium periodate in saline were mixed and left at room temperature for 10 min. M/600 periodate was found to be optimal.

Tissue culture fluid containing Pi-3 virus was then added to give at least 13,500 hemagglutinating units (Compton isolate) or 1.3×10^9 plaque-forming units (J121 isolate) per 0.1 ml of packed erythrocytes. The mixture was left at room temperature for a further 20 min to allow irreversible virus attachment to receptors oxidized by the exposure to periodate. A small amount of hemolysis was always observed at this stage. The treated cells were washed

three times in barbitone-buffered saline, finally packed, and resuspended in barbitone-buffered saline to give a 50% (vol/vol) suspension.

Agarose (1%) (Indubiose A37, L'Industrie Biologique Francaise SA) in barbitone-buffered saline containing 0.1% sodium azide was melted and held at 45 C. A 0.1-ml amount of the treated erythrocyte suspension, 0.2 ml of guinea pig serum (source of complement), and 2.7 ml of the agarose were rapidly mixed and poured into an immunodiffusion plate (Hyland Div., Travenol Labs Inc., Costa Mesa, Calif.).

Wells (3 mm diameter) were punched in the gel and each well was filled with 10 μ l of serum or nasal secretion. The gels were stored at 4 C overnight in a moist box to allow radial diffusion of antibody and then incubated at 37 C for 2.5 h to develop zones of complement-mediated erythrocyte lysis around the antibody-positive wells (Fig. 1).

At least three radii at right angles were measured for each zone with a magnifier incorporating a "cross wire" graticule calibrated in 0.1-mm divisions (Graticules Ltd., Tonbridge, Kent). The mean radius was used to calculate zone area which was corrected by subtraction of the well area.

HI antibody assay. The microtiter method described by Rossi and Kiesel (12) was used except that the plates were incubated at 37 C for 45 min after the addition of 1 drop (0.025 ml) of 1% human "O" erythrocytes to the virus/serum mixture. The Compton isolate of Pi-3 virus was used in the assay and all sera were absorbed with human O erythrocytes for 1 h at room temperature before use.

SN test. The microtiter method of Rossi and Kiesel (12) was followed except that secondary calf kidney cells were used.

RESULTS

Although initial experiments utilized the Compton isolate of Pi-3 virus, the J121 isolate was found to be more efficient in SRH and was therefore adopted for the routine assay.

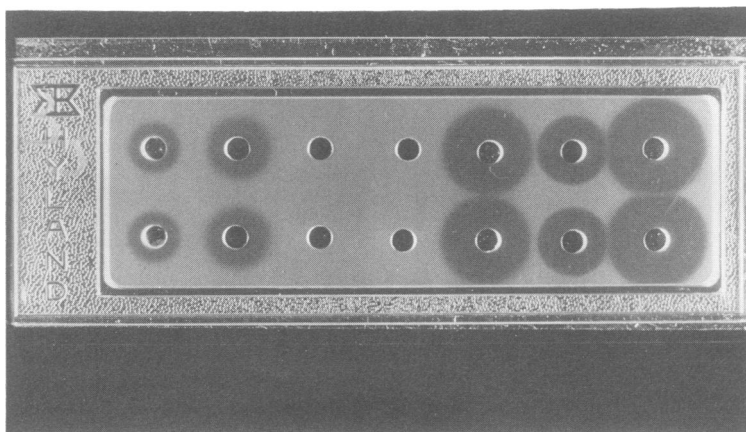


FIG. 1. Hemolysis zones produced by calf nasal secretions (left-hand wells) and sera (right-hand wells) in gels containing J121 Pi-3 virus-sensitized bovine erythrocytes.

Effect of serum dilution and virus concentration on SRH zone area. Treating the erythrocytes with increasing amounts of virus caused a reduction in SRH zone area until a concentration was reached above which no further decrease in zone area occurred (Fig. 2). This was therefore the minimum concentration required to ensure reproducibility and was utilized in all subsequent assays with that particular virus preparation. Figure 2 also shows that an almost linear relationship exists between zone area and the logarithm of serum dilution. The gradients of dose-response curves obtained with several bovine sera were all very similar.

Precision and resolution of the SRH assay. The variation of SRH zone area within and between single gels from a given batch was

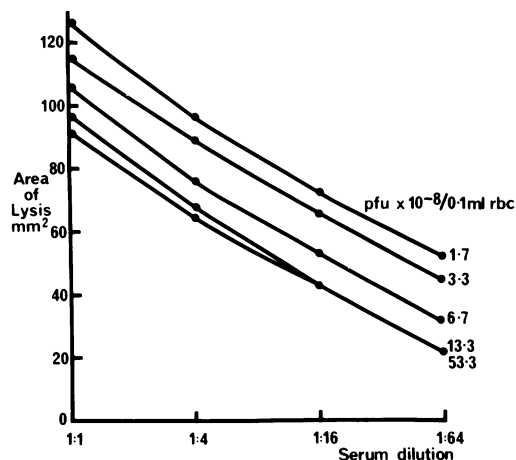


FIG. 2. The effect of serum dilution and virus concentration on SRH zone area (J121 Pi-3 virus used in SRH test; each point represents the mean of four replicate estimations).

investigated and the results are presented in Table 1. It can be seen that the overall variation is of a low magnitude. As expected, the variation in zone area between separate assays carried out on different days was greater (Table 2), but the overall standard deviation only just exceeded 10% of the mean in the case of the smallest zones.

The assay of closely spaced serum dilutions (Table 3) demonstrated the ability of the method to resolve a relative dilution of 1:1.33 (mean zone areas significantly different by the Student's *t* test, $P < 0.01$).

TABLE 1. Variation in area of hemolysis within and between gels from a single batch^a

Gel	Mean area ^b of hemolysis (mm ²)	Standard deviation
1	46.19	1.94
2	46.63	2.16
3	46.62	1.34
4	46.62	1.34

^a Compton Pi-3 virus used in SRH test.

^b Mean areas calculated from six replicate wells of calf antiserum per gel. Overall mean area = 46.51 mm²; overall standard deviation = 1.63 mm².

TABLE 2. Variation in area of hemolysis between separate assays^a

Serum dilution	Calf serum		
	512/21	817	818
Neat	100.75 ^b (5.40)	95.05 (3.94)	46.71 (2.35)
1:4	69.40 (4.80)	67.28 (4.31)	
1:16	42.15 (3.14)	40.30 (2.83)	
1:64	20.91 (2.48)	15.30 (2.06)	

^a Compton Pi-3 virus used in SRH test.

^b Overall mean area (mm²) from four separate assays carried out on different days; standard deviation in brackets. Four replicates per serum dilution per assay.

TABLE 3. Resolving ability of the SRH assay^a

Serum ^b dilution	Mean area of hemolysis ^c (mm ²)	Standard deviation
1:5	63.09	1.49
1:10	49.02	1.32
1:15	42.48	1.24
1:20	35.39	2.22
1:25	31.44	1.80
1:30	27.15	0

^a Compton Pi-3 virus used in SRH test.

^b Calf serum 512/21 (Table 2).

^c Means of four replicates, one replicate per gel.

Correlation of SRH with HI and SN and effect of virus isolate on SRH zone area. A comparison was made between HI titers and SRH zone areas for several groups of calf sera. A highly significant positive correlation between the results of the two assay methods was obtained in a group of 30 sera (Fig. 3). Two sera with HI titers of 20 and 80 produced nonmeasurable, diffuse zones of hemolysis and were scored as SRH zone negative in Fig. 3. However, with the J121 isolate of Pi-3 virus sharper measurable zones were obtained with these sera (21.21 and 35.94 mm², respectively).

In a further group of 48 sera from experimental calves there was a highly significant correlation between the results of HI and SRH and SN and SRH (SRH/HI, $r = 0.93$; SRH/SN, $r = 0.88$, $P < 0.001$). There was also a highly significant correlation between the HI titers and SRH zone areas of 48 parallel nasal secretion samples from the same calves ($r = 0.83$, $P < 0.001$).

HI titers and SRH zone areas in a group of 36 high-titer calf sera (29 sera with HI titers > 160) showed a highly significant correlation which was independent of the virus isolate used for SRH (Compton isolate: $r = 0.64$; J121 isolate: $r = 0.66$, $P < 0.001$). A comparison of the SRH zone areas produced by the two virus isolates (Fig. 4) demonstrated that J121 virus-sensitized erythrocytes gave larger zones. J121 was therefore subsequently used routinely for Pi-3 SRH.

Detection of seroconversions. Upon consideration of the precision of the SRH assay described above, a conservative value of 10 mm² was arbitrarily fixed as the minimum increase

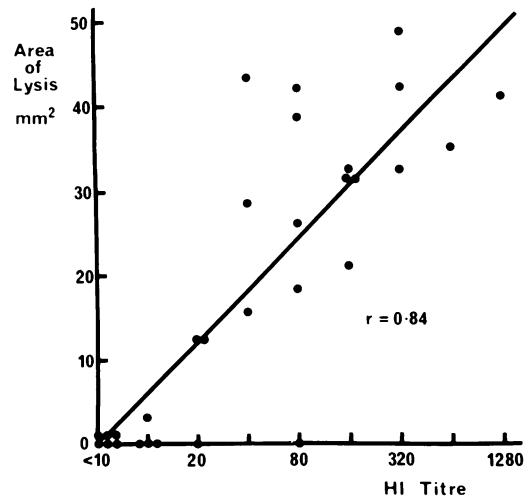


FIG. 3. Correlation between SRH zone area and HI titer in a group of 30 calf sera (Compton Pi-3 virus used in SRH test).

in zone area corresponding to positive seroconversion.

In an experimental Pi-3 trial, 15 calves were challenged with Pi-3 virus and 9 remained as unchallenged controls. Serum and nasal secretion samples were taken at the time of challenge and at 3 weeks postchallenge. Seroconversion of the 15 virus-challenged animals was detected by both SRH and HI. Only 12 seroconversions were detected by SN. In addition, conversion of nasal secretions to antibody positivity was detected in 10 of the challenged animals by HI and in 12 by SRH.

Fractionation of a hyperimmune Pi-3 virus antiserum. The globulin component of a hyperimmune calf Pi-3 virus antiserum was precipitated with 40% saturated ammonium sulfate and the resuspended pellet was dialyzed against barbitol-buffered saline and applied to a Bio Gel A-1.5 million (Bio-Rad Lab., Richmond, Calif.) column. The eluted fractions were

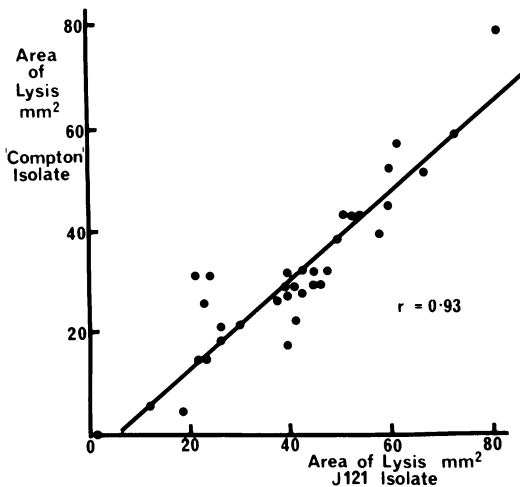


FIG. 4. Correlation of SRH zone areas produced by the Compton and J121 virus isolates in a group of 36 calf sera.

tested for Pi-3 virus antibody by HI and SRH, and the peaks of activity by both assays corresponded to the location of immunoglobulin G (IgG) in the elution profile of the serum (Fig. 5). This result also reinforced the previous observation of close correlation between the HI and SRH tests.

DISCUSSION

The SRH technique (13) has been successfully applied to the assay of Pi-3 virus antibody in serum and nasal secretions. The assay is at least as sensitive as HI and has the major advantage of high precision and accuracy related to the measurement of a continuous variable. It is simple and rapid in comparison to SN tests which require 6 days of incubation and the use of live tissue culture cells (12).

Conversions to antibody positivity in serum and nasal secretions are readily detected and small increases in antibody levels can be reliably measured. These highly significant increases are much less than the minimum 4-fold titer rises required in HI and SN tests. Thus the SRH assay is well suited to the rapid screening of sera and nasal secretions which can be applied to the gels undiluted to give an accurate estimate of total antibody content. Use of a control standard serum would minimize the already small effects of test-to-test variation.

From the results obtained with two separate isolates of Pi-3 virus in the SRH test it is apparent that, while the test does not provide evidence for an antigenic difference, the isolate utilized may effect assay sensitivity. The J121 isolate with a weak hemagglutinin activity produced larger, more distinct zones than the Compton isolate which has a strong hemagglutinin activity. Therefore strains with a weak hemagglutinin activity may be more suitable in the SRH assay.

It has been shown that in a hyperimmune serum antibody activity measurable by HI and SRH resides in the IgG fraction. However, fur-

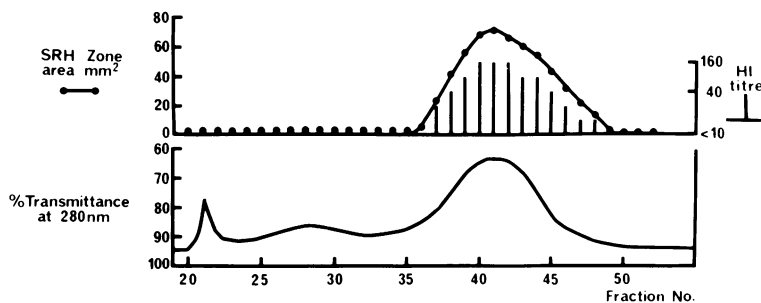


FIG. 5. Relationship between protein concentration, SRH zone area, and HI titer of fractions of a hyperimmune calf Pi-3 virus antiserum eluted from Bio Gel A-1.5 m.

ther studies are in progress on the immunoglobulin species active in early immune sera and nasal secretions. Previous work (8, 9) showed residence of almost all anti Pi-3 virus activity in the IgA fraction, but since IgA does not fix complement (3) the antibody detected by SRH in nasal secretions is thought to be IgG. Preliminary observations suggest that nasal secretions from young calves with high levels of passively derived colostral antibody (largely IgG₁; 4, 11) produce clear SRH zones in contrast to incomplete hemolysis-produced samples from older calves with actively acquired antibody. Secretory IgA could have a blocking effect in the SRH test.

We believe that the SRH assay for Pi-3 virus antibody is a significant improvement on current methods of assay. It should make a contribution to the accuracy, precision, and discrimination of future serological studies with this virus and may even provide information on different components of the humoral immune response.

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