From the State Bacteriological Laboratory and the Department of Virus Research, Caroline Institute, School of Medicine, Stockholm

Hemagglutination-inhibition Test for Titration of Antibodies against Hepatitis contagiosa canis (Infectious Canine Hepatitis)

By

J. Å. Espmark and **C. R. Salenstedt**
with the technical assistance of *Marianne Wallin*

With 2 Figures

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Routine titration of antibodies against hepatitis contagiosa canis (HCC) has until now been performed as complement fixation (CF) or serum neutralization tests (NT). However, these methods have some disadvantages. The CF test is proportionally rather laborious if not included in a large scale run. Furthermore, according to our experience some dog sera will give non-specific fixation with control antigen. The neutralization test, requiring a fairly long observation period, is not particularly well suited for diagnostic service purposes.

An indirect hemagglutination-inhibition test with tanned cells has recently been described by *Carmichael* and *Sarkar* (1). The delicate tanned cell technique calls for specially trained personnel and it is also rather time-consuming.

Fastier (2) and *Mantovani* and *Gramenzi* (3) obtained a direct hemagglutination (HA) with HCC virus. *Fastier* tested the agglutinability of erythrocytes from ordinary laboratory animals and from man, finding only fowl red cells to be specifically agglutinated, while *Mantovani* and *Gramenzi* reported positive results with erythrocytes from a large number of different animals species including human and fowl red cells. As far as we have found no one has as yet devised a useful direct hemagglutination inhibition (HI) test for routine antibody titration.

In the attempts to investigate some basic conditions for applying the direct HI test in HCC antibody titration we obtained results which were partly in conflict with those of *Fastier* and the Italian authors.

The purpose of the present work was to design a simple and useful HI test for titration of HCC antibodies and to discuss some of the underlying experimental conditions.

Material and Methods

HA-antigen: The strain SBL: S I (4) of HCC virus of the 3rd laboratory passage was used to inoculate dog kidney monolayer cultures maintained in Parker's medium 199. When general cell degeneration had occurred the whole cultures were frozen and thawed twice and distributed in 1 ml. portions in rubber stoppered glass tubes. This stock antigen was kept frozen at $-20°$ C until immediately before use. The infectivity titer of the stock antigen was $10^{7.3}$ ID_{50} in dog kidney tissue cultures. A control antigen was prepared in a similar manner from non-infected dog tissue cultures except that freezing and thawing was repeated four times.

The identity of the used virus strain has earlier been tested by *Salenstedt* (4) in cross neutralization experiments against four American HCC-strains, no immunological differences being observed. A further check was made in the present study where guinea pig immune serum against the used strain was titrated in HI-test against the homologous antigen, against the two mentioned American strains and against two virus strains recently isolated in our laboratory from the livers of dogs with histopathologically verified HCC-diagnosis. The positive serum gave similar inhibition titers against all the tested HCC-strains.

Erythrocytes: Human 0 group blood was collected in ACD solution once weekly and stored in the refrigerator ($+4°$ C). Before use the blood cells were spun down and then washed 3 times in 10 volumes of isotonic saline. For the tests a 0.25 per cent suspension in saline was prepared from the packed erythrocytes.

Treatment of sera for HI tests: All sera were inactivated for 30 min. at $+56°$ C. On certain occasions it was found necessary to absorb sera with packed erythrocytes or kaolin to eliminate non-specific "agglutinins" or non-specific inhibitors.

Erythrocyte-absorption: 0.1 ml. of packed 0 group human blood cells was added to 0.1 ml. of undiluted serum in a small centrifuge tube. After 20 min. at room temperature 0.9 ml. of saline was added to the tube and the erythrocytes spun down in an angle centrifuge. The supernatant was considered as serum diluted 1 : 10.

Kaolin-absorption: Washed kaolin powder (5) (Amend Drug & Chemical Co., New York City) was made up to a 25 per cent suspension in saline. 0.5 ml. of kaolin suspension was added to 0.5 ml. of serum diluted 1 : 5 in saline. The mixture was kept at room temperature for 20 min. under occasional shaking. The kaolin was spun down in an angle centrifuge (3000 rpm. in 20 min.) and the supernatant considered as serum diluted 1 : 10.

Hemagglutination test (= antigen titration): All tests were made in round-bottomed glass tubes (10×80 mm.) set up in wire-net racks. 0.5 ml. volumes of 2-fold antigen dilutions in saline were prepared, the dilutions usually ranging from 1 : 10 to 1 : 5120 (10 tubes). An equal volume of a 0.25 per cent human 0 blood cell suspension was added, the racks shaken and left for 2 hours at room temperature before reading. Controls were set up with a 1 : 10 dilution of control antigen and with saline, respectively,

instead of virus antigen. Results were determined from the sedimentation pattern which was read and recorded as generally practised in influenza hemagglutination tests (6). Titers were given as the highest antigen dilution causing complete or partial hemagglutination.

Hemagglutination-inhibition (HI) titrations: To each of 10 tubes with 0.2 ml. amounts of serial 2-fold dilutions of the treated serum, starting with 1 : 10, were added 4 HA-units of antigen in a 0.2 ml. volume and after shaking 0.4 ml. of the erythrocyte suspension. A test antigen titration and, for each serum one tube with serum diluted 1 : 10 + saline instead of antigen, were run as simultaneous controls. Readings were taken as above and the HI-antibody titer was given as the last serum dilution which completely inhibited the viral hemagglutination.

Complement fixation (CF) and neutralization tests (NT) were performed according to conventional methods as reported in an earlier paper by *Salenstedt* (4). In both reactions the source of antigen was the same as in the HI-tests. In CF titrations the first serum dilution was 1 : 5 or 1 : 10. Neutralization tests were made in dog kidney tube cultures starting with a serum dilution of 1 : 8.

Experimental

Standardization of the experimental conditions

Erythrocytes. Red blood cells from cynomolgus monkey and adult fowl were not agglutinated at all by the HCC-antigen used. With erythrocytes from new-hatched chickens and guinea pigs irregular and very low titers were obtained. The only practically usable cells were found to be albino rat and human 0 group erythrocytes, which both gave approximately similar titers ranging from 1:320 to 1:1280. A considerable proportion of the rat blood batches were agglutinated spontaneously either in plain saline or in the presence of dog serum as indicated by positive serum controls in HI tests. Furthermore the rat cells needed 4—5 hours to settle in the tubes to give a good pattern. These initial experiences soon led to the abandoning of rat cells for routine titrations, human 0 group erythrocytes being finally adopted. Thirteen human 0 red cell samples obtained from different blood donors were tested simultaneously in HA-titrations with HCC antigen. All cell samples gave practically identical titers the specificity of which was checked by inhibition with homologous immune serum. There was no agglutination noted in control tubes with saline or control antigen.

Influence of pH and temperature on HA titers was studied in an experiment summarized in Table 1. As can be seen the HA titers were largely unaffected by reasonable variations in the test conditions mentioned. Consequently isotonic saline could be adopted as diluent for all included reagents and the titrations could be made throughout at room temperature.

Nonspecific substances in sera. Factors in sera which might adversely affect the specificity of the HI reactions are non-specific "hemagglutin-

ins" and inhibitors of non-antibody character. Of 33 dog sera tested no one gave nonspecific hemagglutination whereas some of the guinea pig sera did to a minor extent. The nonspecific agglutination of the guinea pig sera could be removed by absorption with packed human erythrocytes. Nonspecific inhibitors were occasionally found in negative dog sera but not in guinea pig sera. The inhibitors in dog sera could be successfully removed by treatment with a 25 per cent suspension of kaolin powder. In accordance with these findings dog sera in routine titrations were treated with kaolin and guinea pig sera were absorbed with erythrocytes. Although no heat-labile inhibitors were observed all sera were inactivated at 56°C for 30 minutes.

Table 1. Influence of different pH and temperature on hemagglutination titers of HCC antigen. 0.25 per cent suspension of human 0 group erythrocytes added. Reading after 2 hours

	pH 6.5	pH 7.0	pH 7.5	pH 8.0
Agglut. temp. +37° C	1/160	1/160	1/20	1/10
Agglut. temp. +37° C	1/320	1/320	1/160	1/10
Agglut. temp. + 4° C	1/320	1/320	1/20	<1/10

Binding time for antigen-serum mixtures. Four replicate titrations of a positive serum were made and constant amounts of antigen added. Erythrocyte suspension was added at different times i. e. immediately, after 10 min., 1 hour and 3 hours. The inhibition titer of the first row (blood added immediately) was one two-fold dilution lower than the 3 following rows which were all alike. Variation within reasonable limits in the length of the binding time was thus considered to be of little importance in routine tests.

Comparison between HI, CF and neutralizing antibodies in sera from HCC-infected guinea pigs

As reported earlier by *Salenstedt* (7) guinea pigs are susceptible to experimental infection with HCC virus. Subsequently this animal was chosen for a study of the antibody development as followed with the 3 different titration methods.

Ten guinea pigs were inoculated subcutaneously each with 1 ml. of a high titering HCC virus suspension. Blood specimens were drawn at different times and then tested simultaneously for HI, CF and neutralizing antibodies. The number of animals with HCC antibodies at the different bleedings as well as average titres of the positive sera are given in Table 2. It shows that the HI test in this case seems to reveal the first traces of

antibodies earlier than the other tests. However, 10 days after infection and thereafter there is no significant difference between the proportion of positive animals as estimated by the three titration methods. Although the incompleteness of the 10 days data may cause an overestimate of the corresponding mean titer values, it seems evident that the average titer

Table 2. HCC antibodies in guinea pigs experimentally infected with HCC virus. Proportion of animals positive in HI, CF and neutralization tests on indicated days after inoculation. Within brackets geometrical mean titers of positive animals

	Time of bleeding, days after inoculation.				
	4 days	7 days	10 days	17 days	21 days
HI tests	1/10	5/10 (1:20)	9/10 (1:17)	10/10 (1:43)	9/9 (1:85)
CF tests	0/10	1/9	6/9 (1:22)	10/10 (1:40)	6/6 (1:45)
Neutralization tests	0/10	0/10	6/9 (1:13)	10/10 (1:224)	9/9 (1:603)

increase from day 10 to day 21 is high for neutralizing antibodies (more than 45×), moderate for HI antibodies (about 5×) whereas CF antibody mean is rising only about two-fold. The location in time of the titer rises is illustrated also by Table 3. For both the HI and CF test about half the

Table 3. Antibody titer rise in HCC infected guinea pigs. Proportion of animals with at least 4-fold rise in HI, CF and neutralizing antibodies during indicated time intervals

	Proportion of animals with titer rises between days:		
	4 and 10	10 and 17	10 and 21
HI tests	5/10	5/10	8/9
CF tests	5/9	5/9	1/5
Neutralization tests	2/9	9/9	8/8

number of animals had a significant titer rise up to day 10, and half the number thereafter. In the neutralization test all tested animals had high titer rises between day 10 and day 17 or 21.

Routine Titrations of Dog Sera

Sera were obtained from dogs with clinical manifestations of various diseases. The sera were inactivated at +56°C for 30 min. and treated with kaolin (cf under Material and Methods). The HCC antibody titre was determined by CF-test. The titre range extended from less than 1/5

to 1/80. From each CF antibody level tested a number of specimens were also examined in HI-test. The results are plotted diagrammatically in Fig. 1. Evidently there is a correlation between the CF- and HI-titers. However, three sera with a CF-titer less than 1/5 had HI-titers of 1/10, 1/20 and 1/80 respectively. The two latter sera were tested in a serum neutralization test against 100 $TCID_{50}$ of HCC virus and were shown to neutralize the virus in titers 1/25 and 1/625. These obviously positive sera missed in the CF-test were thus picked up in the HI titration.

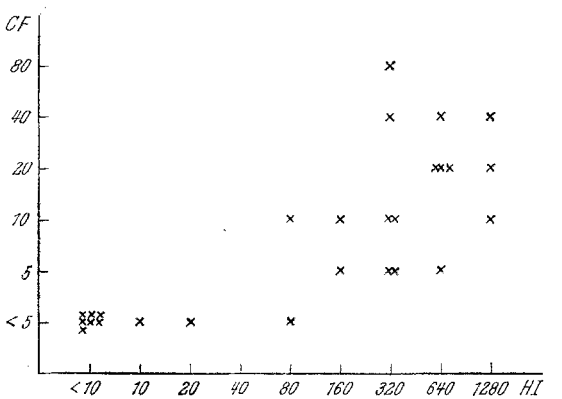

Fig. 1. HCC antibodies in dog sera titrated by complement-fixation (CF) test (ordinate) and hemagglutination-inhibition (HI) test (abscissa).

A modification of the HI test for antibody screening

It has been shown for many viral HI-systems—e. g. by *Hirst* in influenza HI experiments (8)—that there is in conventional HI-test a direct relationship between the inhibition titer of a serum and the dilution of the antigen used. From Table 4 it can be seen that a similar relationship is valid, at least approximately, also for the HCC-system. The HI antibody content of a serum may thus be roughly estimated by adding a constant serum concentration to a dilution series of the antigen.

Table 4. Chess-board titration with 2-fold dilutions of a HCC-positive guinea pig serum and HCC antigen

		Dilution of serum								
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	Saline
Dilution of antigen	1/5	—	—	+	+	+	+	+	+	+
	1/10	—	—	—	+	+	+	+	+	+
	1/20	—	—	—	—	+	+	+	+	+
	1/40	—	—	—	—	∓	+	+	+	+
	1/80	—	—	—	—	—	+	+	+	+
	1/160	—	—	—	—	—	∓	+	+	+
	1/320	—	—	—	—	—	—	—	+	+
	1/640	—	—	—	—	—	—	—	—	—

A similar method has been used, among others, by *Sigel* et al. (9) in screening human sera for influenza antibodies.

To test the reliability of such a test device the following titration was performed on the 28 above-mentioned dog sera already tested in an ordinary HI-test.

Ten 2-fold dilutions from 1/5 to 1/2560 in saline of HCC antigen in large volumes were prepared and 0,2 ml. portions transmitted to corresponding tubes in 30 ten-tube rows, resulting in identical antigen dilution

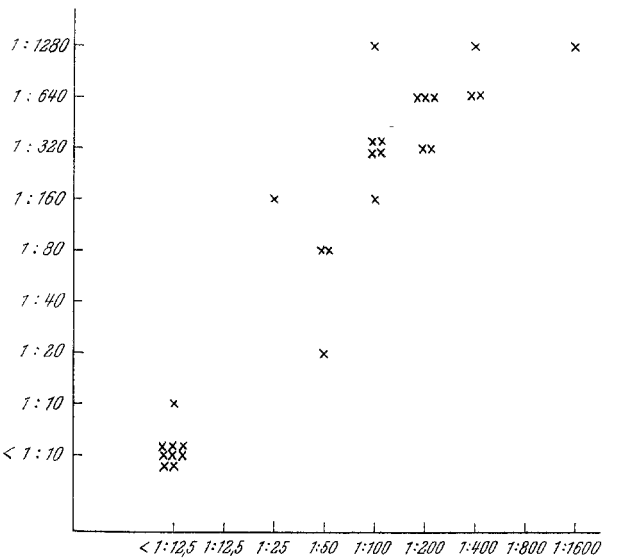

Fig. 2. Comparison between HI titers obtained by two different test methods. The experimental series consisting of 28 dog sera. Ordinate: Conventional method. Abscissa: Screening method.

series for the 28 test sera and for two controls. To each tube in a row was then added 0,2 ml. of kaolin-treated test-serum diluted 1:50. The two control rows were given 0,2 ml. of a similarly treated normal serum and saline respectively. After adding 0,4 ml. of 0,25 per cent human 0-group erythrocytes per tube and leaving the tubes for 2 hours at room temperature, the agglutination end-point titer of each row was estimated as already mentioned.

In interpreting the titration result the end-point titers of rows with test serum were compared with the titers of controls. If a serum reduced the HA titer by 3 steps as compared with the controls, i. e. inhibited 4 HA units of antigen, the HI titer of the serum was considered to be equal to the serum dilution used—in this case 1:50. A reduction of the HA titer by 4 steps would give a two-fold higher HI titer etc.

Some strong sera which did inhibit all tubes in the row were submitted to a repeated similar test using dilutions 1:400 of the sera. In Fig. 2 the antibody estimates obtained by this "constant serum" method are plotted against the titer values from HI test of conventional type. Apparently the correlation is good enough to justify the alternative recommendation of the "constant serum" method for HCC antibody screening in dog sera.

Discussion

The hemagglutination-inhibition (HI) test for HCC antibody titration seems to be a satisfactory alternative for the hitherto used CF and neutralization tests. Our experience has been that the method involves few sources of error and thus that repeated tests on the same sera will seldom be required. It is at the same time simple and sensitive. Thus in our material two sera negative in CF test were positive in the HI titration as well as in the neutralization test. However, there is of course no evidence of identity between CF and HI antibodies. The HI test is apparently well suited for laboratories with limited resources.

Apparently some of our results are in conflict with those of *Fastier* (2) and *Mantovani* and *Gramenzi* (3). *Fastier* obtained agglutination only with fowl red cells and the optimal HA conditions were pH 7,5—8,0 at +4°C. He obtained no agglutination at 37°C and could furthermore show elution at that temperature of virus from the erythrocytes already agglutinated at lower temperatures. In our experiments only rat and human 0 group erythrocytes were agglutinated to satisfactory titers while red cells from adult fowl did not agglutinate under any test conditions. We found (Table 1) that the HA titer was not significantly influenced by variation in temperature conditions and there was no obvious evidence of virus elution at +37°C. Nor was the HA seriously affected by pH fluctuations in the interval 6,5—7,5. The ratio between the infectivity titer and the HA titer of the antigen amounted to 10^4—10^5.

The observation of the wide range of animal species with agglutinable red cells reported by *Mantovani* and *Gramenzi* (3) could not be confirmed by us.

The mentioned disagreements between our results and those of the above mentioned authors cannot be satisfactorily explained. Some of the divergences may however be attributed to different stages of virus adaptation in the laboratory host systems.

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