past 10 years has been 1 in 20,400 births.

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## Direct Complement Fixation by Turkey and Chicken Serum in Viral Systems.*† (22881)

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Bushnell and Hudson(1) used both complement fixation and agglutination procedures for diagnosis of *Salmonella pullorum* in chicken and turkey serum. They were able to get fixation of complement with unheated serum only. The failure to produce reactions with inactivated serum was attributed to destruction of the antibody by heat. In addition they stated that concentration of serum greater than 1/50 could not be read because of the anti-complementary quality of the serum. Rice(2) repeated this work and encountered essentially the same difficulties. She did not attribute the inability of heated serum to fix complement to destruction of antibody, but rather to the presence of inhibitory or compensatory factor in certain fowl sera. The result of this work was the development of the indirect complement fixation method(3).

In the following procedure unheated turkey and chicken sera are used in direct complement fixation. The test is qualitative in nature, designed for diagnostic work where large numbers of specimens are involved. Three viral systems have been tested, ornithosis, Newcastle disease and infectious bronchitis.

*Materials and methods.* The methods for direct and indirect complement fixation were essentially those of Hilleman, Haig and Helmold(4), *i.e.* all reagents were adjusted to 0.2

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TABLE I. Turkey Serum Titers in Direct and Indirect CF Psittacosis Antigen.

| Flock # | Sample No. | CF | CFI | Flock # | Sample No. | CF | CFI | Flock # | Sample No. | CF | CFI |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | ac | ac | 3 | 55 | <1/ 8 | >1/64 | 3 | 68 | <1/8 | 0 |
| | 6 | 1/ 8 | 1/ 8 | | 56 | <1/64 | 1/16 | | 69 | " | 0 |
| | 19 | " | " | | 57 | " | 1/32 | | 71 | 1/8 | <1/ 8 |
| | * | 0 | 0 | | 59 | <1/ 8 | <1/ 8 | | 73 | <1/8 | >1/64 |
| 2 | 26 | ac | ac | | 60 | 1/32 | <1/32 | | 74 | " | 0 |
| | 30 | <1/ 8 | 0 | | 61 | <1/32 | 1/32 | | 75 | " | <1/ 8 |
| | 33 | 1/ 8 | 0 | | 62 | 1/ 8 | 1/ 8 | | * | 0 | 0 |
| | * | 0 | 0 | | 64 | <1/32 | 1/16 | 4 | 88 | <1/8 | <1/ 8 |
| 3 | 52 | ac | ac | | 65 | >1/64 | >1/64 | | * | 0 | 0 |
| | 53 | 1/32 | <1/32 | | 66 | 1/32 | 1/32 | 5 | 101-125 | 0 | 0 |
| | 54 | <1/32 | " | | 67 | " | " | | | | |

Symbol < indicates reaction of 2 or 3 plus at level indicated.
* Remainder.

ml aliquots including 2% cell suspension sensitized with 2 units of hemolysin.‖ The total volume in each tube was one ml. Incubation was carried out at 37°C. The following modifications were made: (a) in place of normal saline, veronal buffered saline pH 7.4(5) containing 0.00015 M $CaCl_2$ and 0.0005 M $MgCl_2$ made with double distilled water was the diluent for all reagents. (b) Both serum and antigen controls received only one-half the amount of complement used in the test and (c) 4 units of antigen were used in the direct test unless it was too anticomplementary or in case of allantoic fluid antigens when the concentrated preparation contained less than 4 units. *Antigens.* There is no fixation of complement in this system with insufficient antigen. The units are determined by titration with avian serum and strong complement explained below. Good results are obtained with a little more than 2 units of antigen, better with 4 units. For the ornithosis system,‡ crude boiled and phenolized preparations of yolk sac antigens of 6 BC strain or Jones strain ornithosis virus were used. The Newcastle antigens were Lederle's diagnostic antigen or allantoic fluid concentrated X5 in the Spinco ultracentrifuge. The infectious bronchitis antigen was also concentrated allantoic fluid which gave satisfactory results with some batches while others were unsuitable. Work is now in progress to produce a stronger and more sensitive antigen for this virus. *Sera.* Where one syringe was used for several bleedings, it was rinsed in saline. The practice of rinsing with an antiseptic caused anticomplementary effects. Serum was removed from the clot as soon as possible after arrival in the laboratory and until this was accomplished the samples were refrigerated at 4°C. In some experiments serum was replaced by citrated plasma with the same results. After samples were cleared of cells they were stored at –20°C. The time elapsing between drawing blood samples in the field and freezing seldom exceeded 24 hours. It has recently been observed that sera stored at 4°C lose their ability to fix complement. Sera for direct procedure were not heated. *Complement titrations.* The complement was pooled frozen guinea pig serum, pretested for absence of reaction with ornithosis antigen. For each new batch, the dilution to be used was established by incubating 0.1 ml (half portion) at dilutions 1/20, 1/22 and 1/24 respectively in the presence of ten unheated avian sera diluted 1/8. The hemolytic system was added after one hour and readings made 30 minutes later. The concentration of complement in 0.1 ml causing hemolysis of the indicator system was considered a half portion. Twice that amount was used for the test. Anticomplementary effects of the antigens were tested by the same method. In this laboratory screening of sera is carried out at 1/8 dilution. Un-

‖ Sharp and Dohme.

‡ We are indebted for the antigens to Dr. David B. Lackman of National Microbiological Inst., Rocky Mt. Laboratory, Hamilton, Mont. and Dr. A. L. Burroughs, Department of Veterinary Bacteriology, Texas A & M College, College Station, Texas.

TABLE II. Effect of Time on Titers of Turkey Sera in Direct and Indirect Complement Fixation.

| CF | | | CFI | | |
|---|---|---|---|---|---|
| 1/25/56 | 6/27 | 7/20 | 1/25/56 | 6/27 | 7/20 |
| neg. | 1/ 8 | <1/64 | neg. | >1/64 | <1/ 32 |
| " | <1/64 | " | " | " | 1/ 32 |
| " | 1/16 | 1/32 | " | " | <1/128 |
| " | 1/ 8 | <1/64 | " | " | " |
| " | <1/ 8 | <1/32 | " | " | >1/ 64 |
| " | 1/64 | 1/32 | " | " | 1/ 32 |
| " | <1/64 | <1/64 | " | " | " |

heated turkey and chicken sera were tested at this level for complement and lysins. It was found that sera frequently lysed unsensitized cells in a dilution of 1/4 and that the same or others often lysed sensitized cells in a dilution of 1/8. However, in the presence of antigen there was no evidence of zoning.

*Results.* The method of random sampling of turkey flocks representing approximately 10% of birds was adopted in a survey for the incidence of ornithosis in turkeys of this region. These birds were double banded and in 5 flocks showing reactors, 25 birds were later spot checked for continuing incidence. Table I shows the results for 125 samples representing 5 flocks. There is agreement between direct and indirect procedures in over 94% of the sera. Titers were higher by indirect in 2% of cases while 4% indicated higher titers or slight reactions at 1/8 dilution by direct. Although there appears to be fair correlation of titers in Table I, there is some evidence that the same type of antibody is not being measured by the 2 methods. In Table II examples of 3 sets of sera for 7 birds demonstrate the shift in titer by direct and indirect procedures as infection progresses.

Newcastle disease antisera resulted from natural infection, vaccination or both. These turkeys and chickens were experimental birds. CF and hemagglutination inhibition titers were run on the same sera. Table III shows fairly good correlation between the two tests. Citrated chicken plasma was used for titrations in Table IV. This indicates that the hemagglutinating inhibitor appears before the complement fixing antibody can be detected.

TABLE III. Turkey Serum Titers in Direct CF and Hemagglutination Inhibition NDV Antigen.

| CF | HI | CF | HI |
|---|---|---|---|
| 1/16 | 1/ 100 | 1/32 | 1/800 |
| <1/32 | 1/ 200 | 1/16 | 1/400 |
| 1/32 | 1/ 400 | " | 1/100 |
| 1/16 | 1/ 200 | " | 1/200 |
| <1/ 8 | " | | |
| 1/64 | 1/1600 | N 0 | 0 |

TABLE IV. Chicken Plasma in Direct CF and HI Tests. NDV antigen.

| CF titer | HI titer | CF titer | HI titer |
|---|---|---|---|
| 2/11/56 | | 2/23/56 | |
| <1/16 | 1/1600 | 1/ 8 | 1/ 400 |
| 0 | 1/ 100 | <1/64 | 1/3200 |
| 0 | 1/ 800 | >1/64 | 1/4000 |
| 0 | 1/ 200 | <1/16 | 1/1600 |
| 0 | 0 | <1/64 | " |
| 0 | 1/ 100 | <1/32 | " |
| 0 | 1/ 200 | >1/64 | " |
| 0 | 1/ 25 | <1/32 | 1/4000 |
| 0 | 1/ 800 | 1/16 | 1/ 800 |
| 0 | 1/ 100 | 1/32 | 1/6400 |

At this time a limited number of sera have been tested in the infectious bronchitis system. It was found that the titer of the allantoic fluid must reach a level of $10^{-7}$ to provide 2 units of antigen. The serum was from birds vaccinated and later challenged with infectious bronchitis virus.

To compare antibody levels, the usual procedure for serum neutralization was reversed. A constant amount of virus, about 100 infectious units, was combined with 2-fold serial dilutions of serum. Table V shows the results for 5 sera.

TABLE V. Chicken Serum in Direct CF and S.N. Tests. Infectious bronchitis antigen.

| CF titer | S.N.* titer |
|---|---|
| <1/ 64 | >1/16 |
| <1/128 | 1/32 |
| 1/ 64 | 1/64 |
| 1/ 16 | 1/16 |
| 1/ 32 | >1/16 |
| N 0 | 0 |

* Approximately 100 ID of virus.

*Discussion.* Heating certain fowl sera appears to release an inhibitory factor which alters or masks the antibody. Rice has suggested(2) the inhibitory factor may be the antibody itself which combines with antigen to form complexes with no affinity for complement. However, it has been observed that

certain turkey sera which originally reacted only by the indirect method, after several months storage, have fixed complement when inactivated. This would suggest a factor somewhat susceptible to cold storage, present in varying quantities in each serum and which in the presence of heat is capable of altering antibody. It does not appear to be free in heated serum. Where equal parts of heated immune or normal avian serum and unheated immune serum were mixed, there was neither increase nor decrease over the antibody titer of the unheated serum alone. Mixing of heated and unheated serum would also indicate that once the antibody had been altered it cannot be restored by unheated serum.

It is recognized that anticomplementary effects of serum and antigen are not additive. With fowl serum they are usually greater than the sum of the two. Proof that fixation resulted from the presence of antibody rather than the combined anticomplementary effects was tested by use of heterologous antigens of the same anticomplementary quality. Immune sera set up against heterologous antigens, within these three systems, gave negative results.

The amount of complement used in the test appears to be large for a viral system. however, the test in practice does not seem to be insensitive. The use of half portions of complement in all controls offers a margin of safety against false or nonspecific reactions.

The inability to demonstrate marked anticomplementary effects in these sera may result from viral disease as opposed to bacterial. Rice has observed(6) that prozones occur more frequently with viral antigens than with bacterial or tissue extract antigens. The level of 1/8 dilution for screening was adopted to minimize anticomplementary as well as possible lytic effects of these sera.

Although correlation of this test with other methods is not perfect, disagreement may result from measuring different types of antibody.

*Summary.* A procedure for direct complement fixation with unheated turkey and chicken serum or citrated plasma is outlined. Three viral systems, ornithosis, Newcastle disease and infectious bronchitis have been tested. Results are compared with other serological methods.

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