J. Hyg., Camb. (1968), **66**, 249
Printed in Great Britain

Serological tests for the detection of antibodies to *Mycoplasma gallisepticum* in chickens and turkeys

By F. T. W. JORDAN and P. KULASEGARAM

Poultry Section, Department of Veterinary Preventive Medicine, University of Liverpool Veterinary Field Station, Neston, Wirral, Cheshire, England

(*Received* 30 *October* 1967)

INTRODUCTION

The relative sensitivity and specificity of the various tests used in the examination of avian sera for antibodies to *Mycoplasma gallisepticum* have been examined by only a few workers. Jungherr, Luginbuhl, Tourtellotte & Burr (1955) compared the whole blood (WB), slide agglutination (SA), tube agglutination (TA) and haemagglutination inhibition (HI) tests on sera from chickens and turkeys naturally infected with *M. gallisepticum*. Hofstad (1957) compared the SA, TA and HI tests on the sera of naturally infected turkeys. Tests were conducted at irregular intervals for a period of 12–21 months, commencing with some birds 2 months after the disease outbreak. Adler (1958) compared the SA and TA tests on the sera of seventy naturally infected turkeys 15 weeks after infection and the sera of sixty-two of them after 27 weeks. Rhoades, Kelton & Heddleston (1965) compared the HI and TA tests on the sera of twenty-four adult turkeys experimentally infected with *M. gallisepticum* and examined their sera at weekly intervals for 11 weeks. Adler & DaMassa (1964) compared the antiglobulin test with the TA test on the sera of five chickens infected experimentally with *M. gallisepticum*, and bled at irregular intervals for 31 days. Baharsefat & Adler (1965) compared the TA, HI and antiglobulin tests on the sera of nine chickens and two turkeys experimentally infected with *M. gallisepticum*. Sera were collected at irregular intervals for 30 days from chickens and 63 days from turkeys.

Leach & Blaxland (1966) showed that the method of preparation of the reactants and the method by which a test is performed will influence its sensitivity and suggested that these factors must be considered when tests are compared, especially when undertaken in different laboratories.

Because it was considered worth while repeating some of these comparative tests and including such other tests as indirect complement fixation (ICF), metabolic inhibition (MI), and gel precipitation (P), giving details of reactants and methods, a series of experiments were undertaken in which sera from chickens and turkeys infected intratracheally with the A514 strain of *M. gallisepticum* were examined.

MATERIALS AND METHODS

Chickens. Brown and White Leghorn chickens were hatched at the Laboratory from eggs from flocks free from the common respiratory pathogens and from resistance-inducing factor (RIF), and were reared in isolation. Cockerels were infected at 4–8 months old, and housed individually in cages; the birds in the different experiments were kept in separate houses.

Turkeys. Broad Breasted White turkeys from a mycoplasma-free flock were reared from 1 day old in isolation. Turkeys of both sexes were infected at 5–10 months of age, and birds in the different experiments were kept in different houses.

Rabbits. English adults were used.

Mycoplasma strain. The A 514 strain of *M. gallisepticum* originally obtained from Dr Chu at Cambridge was used. It had undergone numerous passages in artificial medium.

Brucella broth medium (B.B.M.). Brucella broth* medium was prepared by reconstituting the broth powder following the manufacturer's instructions with the addition of 15% heat-inactivated swine serum, 1% of 1/20 solution of thallous acetate, 50,000 units penicillin (crystalline sodium salt) per 100 ml. and 1% Andrade's indicator.

The pH of the broth was checked and in our experience was always about 7·4. It was filtered through a Seitz EK filter, stored at 0–5° C. in a refrigerator and used within a month of preparation.

Chicken infusion medium (C.I.M.). A modification of the method of Hofstad & Doerr (1956) was used. To prepare 100 ml., 50 g. of chicken meat removed from the breast and legs and liver and heart was cut into small pieces or minced, added to 100 ml. of glass-distilled water and kept overnight in the refrigerator. Next morning it was boiled for 20 min. in a water-bath and filtered through No. 1 Whatman filter paper to remove gross particles. To 80 ml. of this infusion were added 20 ml. of heat-inactivated chicken serum, 1·0 ml. of a 1/20 solution of thallous acetate, 50,000 units of penicillin, 1·0 ml. of Andrade's indicator, 0·5 g. sodium chloride, 0·2 g. glucose (bacteriological), and 1·0 ml. of 1% solution of nicotinamide-adenine dinucleotide. The pH was adjusted to 7·4 with 10% sodium hydroxide, and it was filtered through a Seitz EK filter and stored at 0–5° C. in the refrigerator. It was used within 1 month of preparation.

Turkey infusion medium (T.I.M.). Prepared as C.I.M. except that turkey meat and serum replaced chicken meat and serum respectively.

Rabbit infusion medium (R.I.M.). Prepared as C.I.M. except that rabbit meat and serum replaced chicken meat and serum respectively.

All media supported the growth of the A 514 strain and when the proportion of inoculum at log phase to medium was 1:50, the culture usually attained log phase in 24 hr.

Sera. Sera from experimental birds were removed from the clot within 24 hr. of bleeding, inactivated by heating at 56° C. for half an hour, and stored at 0–5° C. They were examined before storage or within 3 months of it.

* Albimi Labs. Inc., Brooklyn, New York.

Serological tests

Agglutination tests

Three agglutination tests were undertaken: WB, SA, and TA tests. Antigen for these tests was prepared from the A514 strain grown in B.B.M. to log phase, tested for sterility, washed three times in phosphate-buffered saline of pH 6·8 (PBS) with 0·25 % phenol and standardized to an opacity of 15 times No. 4 'Wellcome' tube. Each fresh batch of antigen was also compared with a previous batch for titre and speed of agglutination by the SA test on known positive sera. If less sensitive than the previous batch it was discarded; none were found more sensitive.

Unstained antigen was used for the SA and TA tests, while the antigen for the WB test was the same antigen stained with aqueous crystal violet to give a final concentration of 1/10,000. It was stored at 0–5° C.

Whole blood test. Stained antigen in 0·04 ml. volumes was pipetted on to a white tile and an equal quantity of blood from the wing vein was added with a standard loop. They were mixed and examined after 2 min. for chicken blood and 3 min. for turkey blood. The temperature of the plate was kept at approximately 65° F. (16·5° C.).

Slide agglutination test. Antigen and serum were mixed in equal volumes (0·02 ml.) on a glass slide and read after 2 min. for chicken sera and 3 min. for turkey sera. A known positive and negative serum were included.

The antigen for the WB and SA tests was periodically agitated between tests to keep the organisms in a uniform suspension.

Tube agglutination test. The antigen was diluted 1/15 with PBS for chicken sera and 1/20 for turkey sera. Doubling dilutions of the sera were made from 1/5 in PBS in agglutination tubes. To 0·5 ml. amounts of each of these dilutions was added an equal volume of the antigen. Undiluted serum was also tested. Controls consisted of a positive and negative serum and a tube containing 0·5 ml. of PBS and an equal volume of antigen. The tubes were shaken, incubated at 56° C. for 2 hr. and kept at 0–5° C. overnight and read. The TA test on hyperimmune rabbit sera was conducted as for chicken sera.

Haemagglutination inhibition test

The antigen for the HI test was prepared as described for the agglutination tests except that the organisms were washed in PBS without phenol, and finally suspended in 50 % glycerol–saline to give a final concentration of organisms 100 times that of the original culture. It was stored at −27° C. and used within 6 months of preparation.

A modification of the method of Roberts (1964*a*) was used for the standardization of antigen and for the test itself. A 0·75 % suspension of chicken erythrocytes for chicken sera and 0·75 % suspension of turkey erythrocytes for turkey sera in PBS was used. All reactants were used in volumes of 0·2 ml. In the standardization of antigen 100 % haemagglutination (HA) was taken as the end-point of reaction, and at titre one haemagglutinating dose of mycoplasma was contained in 0·2 ml. Four haemagglutinating doses of antigen standardized in this way were used in the HI test, in which complete inhibition of haemagglutination was the end-point. Initial

work showed that non-specific inhibition which occurred with 4 HA units of antigen standardized to 50% HA was prevented by using 4 units of antigen standardized to 100% HA.

For the HI test serial doubling dilutions of serum were prepared from 1/5 in PBS. Undiluted serum was also tested. Antigen and cell controls were included and readings were taken when settling had occurred in the cell control well.

The indirect complement fixation test

In addition to the titration of complement and haemolytic serum, rabbit antiserum prepared against *M. gallisepticum* was titrated in a CF test against *M. gallisepticum* antigen to determine optimal proportions. The antigen was prepared as for the agglutination test but subjected to ultrasonic disintegration.

The ICF test was performed as a 3-day test. On the first day a twofold series of dilutions* was made of each serum, from 1/1 to 1/1024, and 0·1 ml. volumes were transferred to wells of a Perspex tray. Each well then received 0·1 ml. of *M. gallisepticum* antigen containing 1 (OPD) unit. The tray was gently shaken and kept at 0–5° C. overnight. On the second day 0·1 ml. rabbit serum containing 2 (OPD) units was added to each well, and then 0·1 ml. containing 2 units of complement. The tray was shaken and kept at 0–5° C. overnight. On the third day the tray was removed and placed in the incubator at 37° C. for 30 min. To each well was then added 0·1 ml. of sensitized 2% sheep red blood cell suspension, the tray was re-incubated for 30 min., shaken occasionally, allowed to stand at room temperature for 1 hr., and readings were taken. The highest dilution of the serum associated with 50% lysis of the red blood cells was considered to be the titre of the serum.

The test included controls for the amount of complement, for non-specific lysis of red cells by diluent, for optimal proportion of antigen and rabbit serum, and anticomplementary activity of the antigen and rabbit serum.

Metabolic inhibition test

Doubling serial dilutions of the serum from 1/2 were made in B.B.M. with 0·002% phenol red as indicator; 0·1 ml. quantities of each dilution of serum and of undiluted serum were transferred to tubes containing 0·5 ml. of B.B.M.; and then 0·2 ml. of a log phase culture of *M. gallisepticum* diluted 1/10 in B.B.M. was added to each tube. The controls for the test included a tube containing 0·6 ml. of B.B.M. inoculated with 0·2 ml. of diluted culture to determine the first appearance of colour change and another tube containing 0·8 ml. of B.B.M. for matching colour. The tubes were sealed with sellotape and incubated at 37° C. until the first colour change was seen in the control. The highest dilution of serum which prevented the colour change at this time was taken as the metabolic inhibition titre of the serum.

Precipitation test

Precipitation tests were undertaken by the double diffusion agar gel technique carried out in Petri dishes. The agar gel contained 1·5% 'Special Agar Noble'

* The dilent used in the ICFtest was prepared from Barbitone, CFT tablets as recommended by the manufacturer (Oxoid Ltd).

(Difco), 8% sodium chloride (1% when testing rabbit serum) and 1% sodium azide. Bentonite (1·5%) and Hyflo-Super cel* (1·5%) were added for clarification (Feinberg, 1956). Wells were cut in the agar as shown in Fig. 1. Two adjacent wells

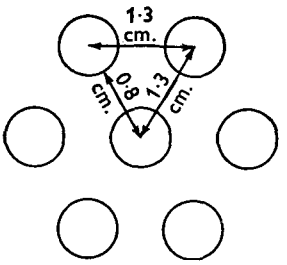

Fig. 1. Size and arrangement of wells in the agar-gel precipitation test.

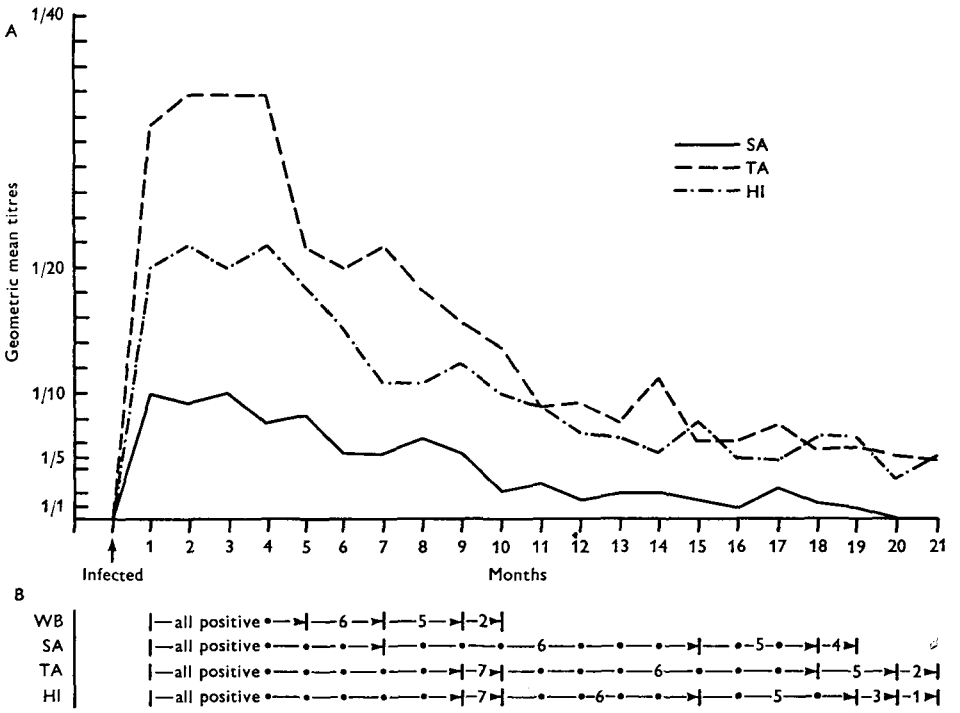

Fig. 2. A. The geometric mean titres of eight chickens inoculated intratracheally with a culture of *M. gallisepticum*. B. The number of positive reactors shown by WB, SA, TA and HI tests.

were used for each serum sample, the plates were incubated at 37° C. in a moist atmosphere for 5 days and examined daily. Antigens were prepared by concentrating a culture in B.B.M. 25, 50 and 100 times and resuspending the organisms in the supernatant. Only undiluted serum was tested.

* Johns-Manville, U.S.A.

https://doi.org/10.1017/S0022172400041115 Published online by Cambridge University Press

RESULTS

Experiment 1

Eight 5-month-old Brown Leghorn cockerels were inoculated intratracheally with 3 ml. of a culture of *M. gallisepticum* grown in B.B.M. containing 10^9 viable organisms per ml. At the time of inoculation and monthly for 21 months, the chickens were bled, the blood examined by the WB test and the sera by the SA, TA and HI tests.

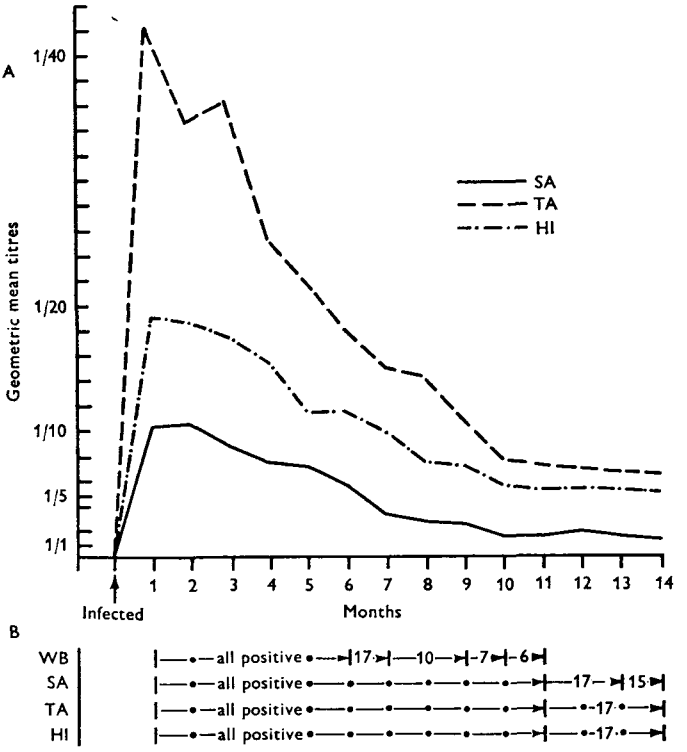

Fig. 3. A. The geometric mean titres of twenty-two chickens inoculated intratracheally with a culture of *M. gallisepticum*. B. The number of positive reactors shown by WB, SA, TA and HI tests.

The geometric mean titres of the sera are shown in Fig. 2. At the time of inoculation all sera were negative by the above tests, but 1 month after infection the sera of all birds was positive by all tests and peak titres were obtained during the first 4 or 5 months.

A similar pattern was shown by all the tests. At first all the birds gave positive reactions but after about 5 months there was a gradual decline in the number with positive sera. The largest number of positive reactors at all periods after infection were obtained with the TA and HI tests while the least number were obtained by the WB test. Highest titres were obtained with the TA test, which were approximately one dilution higher than the HI titres and two dilutions higher than the SA

titres during the first 5 months. After this there was little difference between TA and HI titres, both of which were about one dilution higher than the SA titres. The titre of the serum from a bird was occasionally found to be higher at one test than at the previous test. However, this was never more than one dilution by any of the tests and there was not a similar pattern of fluctuation shown by all three tests on the same serum. Once a bird had shown a negative reaction by a test it never subsequently showed a positive reaction by the same test.

Experiment 2

Experiment 1 was repeated with twenty-two 1-year-old Brown Leghorn cockerels which were inoculated intratracheally with 3 ml. of *M. gallisepticum* culture grown in C.I.M. containing 10^8 viable organisms per ml. At the time of inoculation and at monthly intervals for 11 months they were bled and the blood and sera examined. Five were now removed for another purpose and the remaining seventeen tested as before for a further 3 months.

The results are shown in Fig. 3 and closely parallel those obtained in Expt. 1.

The highest titres were obtained with the TA test, followed by the HI test, and all seventeen birds were positive reactors to these tests when the experiment ended. Lower titres were obtained with the SA test and two of the seventeen birds were negative at 14 months.

After 6 months fewer reactors were detected by the WB than by the other tests and none after 11 months.

Experiment 3

In order to study the antibody response to infection within the first month of inoculation and to compare additional serological tests, twenty-six 10-month-old chickens were inoculated intratracheally with 3 ml. of *M. gallisepticum* culture grown in C.I.M. containing 10^{10} viable organisms per ml.

In addition to agglutination and HI tests the sera were examined by the ICF, MI and precipitation tests. The chickens were bled at the time of inoculation and every 2 days for 10 days, when they were divided arbitrarily into two groups, the groups being bled alternately, so that individual birds were bled every 4 days. This was continued for a month, after which all the birds were bled at monthly intervals until the termination of the experiment after 5 months. One bird died 3 months after inoculation.

The results are shown in Fig. 4. Positive titres were obtained from some sera by all except the WB and P tests by as early as the 4th day and all birds were positive to all the tests except the precipitation test by the 8th day. By the 10th day maximum titres were attained; the highest with the ICF test and the lowest with the MI test. The same pattern was shown by agglutination and HI tests as in earlier experiments. At the end of the first month titres obtained with the ICF test had fallen below those seen with the TA test and at 2 months twenty-three birds had positive sera, but none were positive after 4 months, whereas sera tested by the TA and HI tests were positive throughout. All the sera were anticomplementary up to a dilution of 1/8.

By the MI test low titres were obtained with all the sera, the highest being 1/8, 6–12 days after inoculation. All were negative after 4 months.

Sera from only five of the twenty-six chickens gave a positive precipitation reaction. All five showed positive reactions with sera taken on the tenth day and some on the eighth and twelfth days, as shown in Table 1.

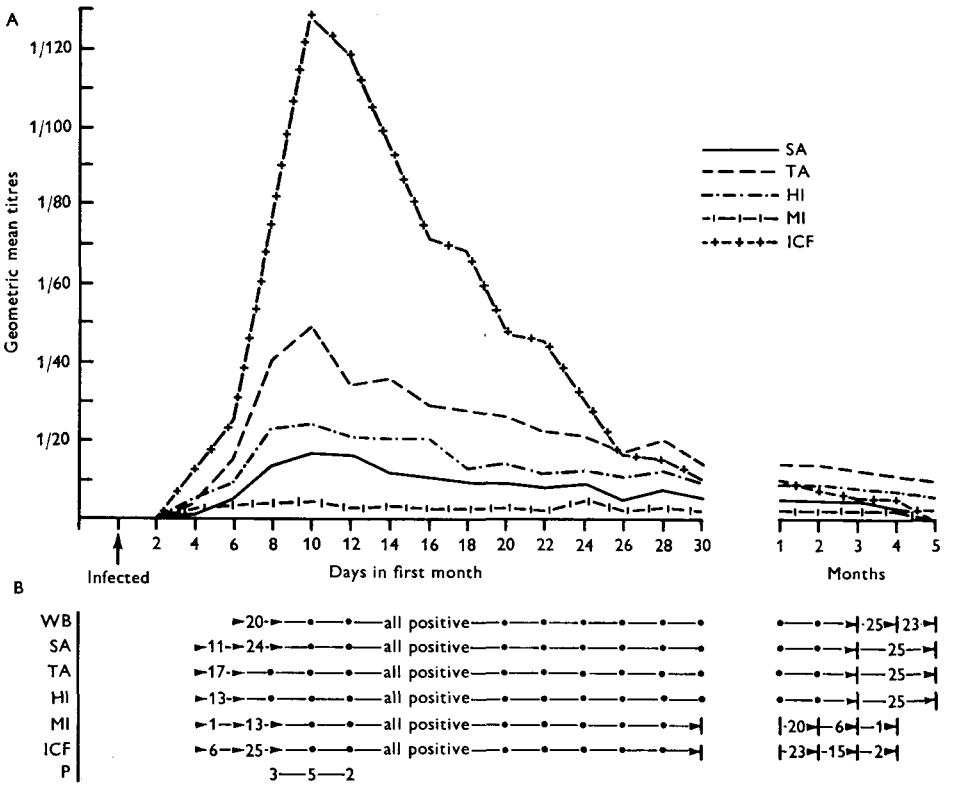

Fig. 4. A. Geometric mean titres of twenty-six chickens inoculated intratracheally with a culture of *M. gallisepticum*. B. The number of positive reactors by the WB, SA, TA, HI, MI, ICF and P tests.

Table 1. *Time of appearance of positive precipitation reactions in five chickens in Expt.* 3

Chicken no.	Days after inoculation		
	8	10	12
1	−	+	+
2	+	+	+
3	−	+	−
4	+	+	−
5	+	+	−

Experiment 4

Twenty-one 5-month-old turkeys were inoculated intratracheally with 3 ml. of a culture of *M. gallisepticum* in T.I.M. containing 10^9 viable organisms per ml. They

https://doi.org/10.1017/S0022172400041115 Published online by Cambridge University Press

were bled every other day for 1 month and monthly for a further 2 months, and the blood and sera examined.

The pattern of results with the agglutination, HI and MI tests (Fig. 5) was similar to that obtained with chicken sera except that peak titres were lower and the period during which positive reactions were obtained was shorter. The highest titre, 1/40, was obtained with the TA test on the sera of four birds, while titres of 1/20 were obtained with the HI and SA tests on the sera of eight and two birds

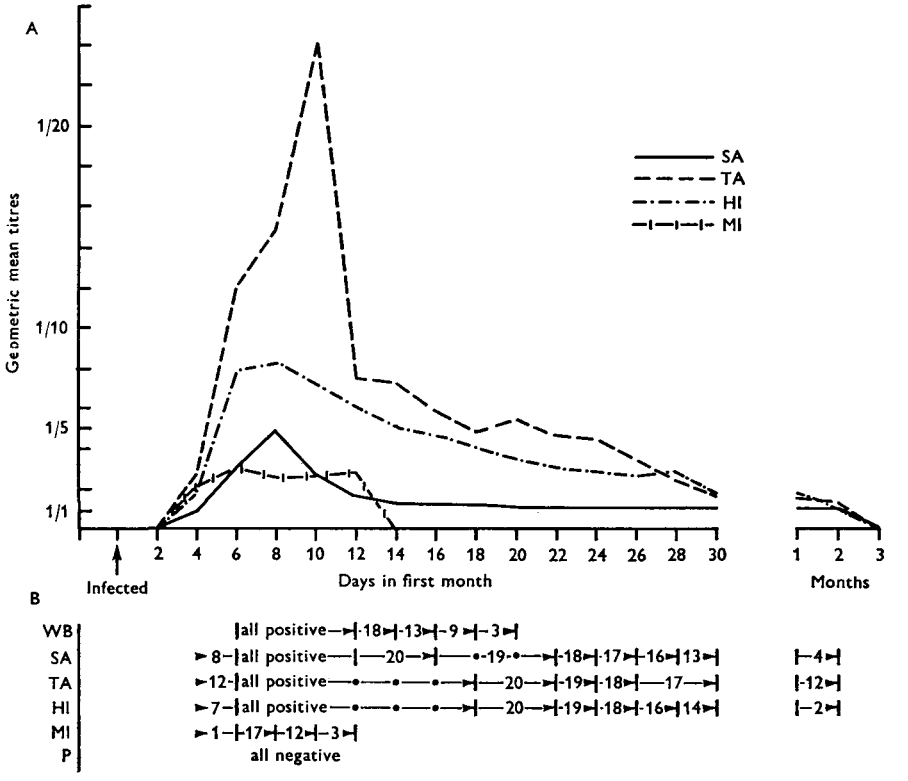

Fig. 5. A. The geometric mean titres of twenty-one turkeys inoculated intratracheally with a culture of *M. gallisepticum*. B. The number of positive reactors by the WB, SA, TA, HI, MI and P tests.

respectively. By all tests the highest titres were observed about 10 days after infection. Afterwards the titre of the sera and the number of birds reacting fell so that 2 months after infection positive titres were shown by the sera of twelve of the turkeys by the TA test, four by the SA test and only two by the HI test. Three months after infection all the sera were negative by all tests. By the WB test all the turkeys gave positive reactions 6 days after inoculation. After the 12th day this number fell and none of the birds were positive by this test after the 20th day. The sera were anticomplementary up to a dilution of 1/16, and with dilutions greater than this there were no positive ICF reactions. By the MI test the highest titre, 1/4, was obtained between the 6th and 10th day after inoculation but no

birds gave positive sera after the 12th day and two did not react at all. No positive precipitation reactions were obtained at any time during the 3 months after inoculation.

Experiment 5

To study the serological response to re-infection with *M. gallisepticum*, ten of the twenty-six chickens from Expt. 3 were housed separately in two groups of five, 5 months after the initial infection. The birds of one group were re-inoculated intratracheally with 3 ml. of a culture of *M. gallisepticum* in C.I.M. containing

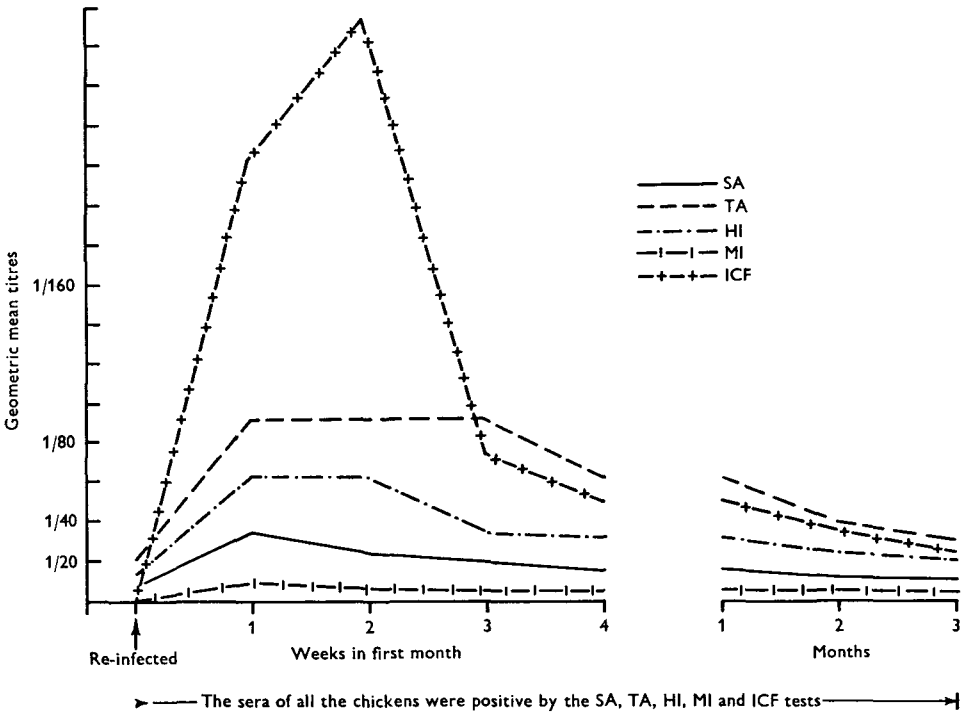

Fig. 6. Geometric mean titres of five chickens re-inoculated intratracheally with a culture of *M. gallisepticum* 5 months after the initial inoculation.

10^{10} viable organisms per ml. At the same time two chickens of the same age but not previously infected with *M. gallisepticum* were similarly inoculated intratracheally and housed with this group. The five birds of the other group were not re-inoculated. All twelve chickens were bled at the time of inoculation, at weekly intervals for 1 month, and then monthly for a further 2 months, and the sera examined.

At the time of re-inoculation the sera of the ten birds from the previously infected group gave positive reactions by the SA, TA and HI tests but antibodies were not detected by ICF, MI or precipitation tests.

The pattern of serological response after re-inoculation is shown in Fig. 6 and followed that obtained after the initial inoculation (Fig. 4). However, titres were higher by one or two dilutions by all tests and persisted at a higher level. Titres by

the ICF test rose and fell rapidly and although the highest titres were obtained with this test, they had fallen by the 4th week below those of the TA test. Titres by the MI test were higher for three of the five birds but the highest titre was only 1/16. By the precipitation test, two lines were obtained with the sera from the five birds at 1 week but not after this time. Positive reactions were obtained for all birds by all except the precipitation test at 3 months, when the experiment ended.

The sera from the five birds which were not re-inoculated showed a gradual fall in titre by the SA, TA and HI tests and no reaction at all by the ICF, MI and precipitation tests. The sera of the other two chickens not previously infected were negative to all tests at the time of inoculation but afterwards gave a positive reaction to all except the precipitation test, the pattern of response being similar to that shown in Fig. 4.

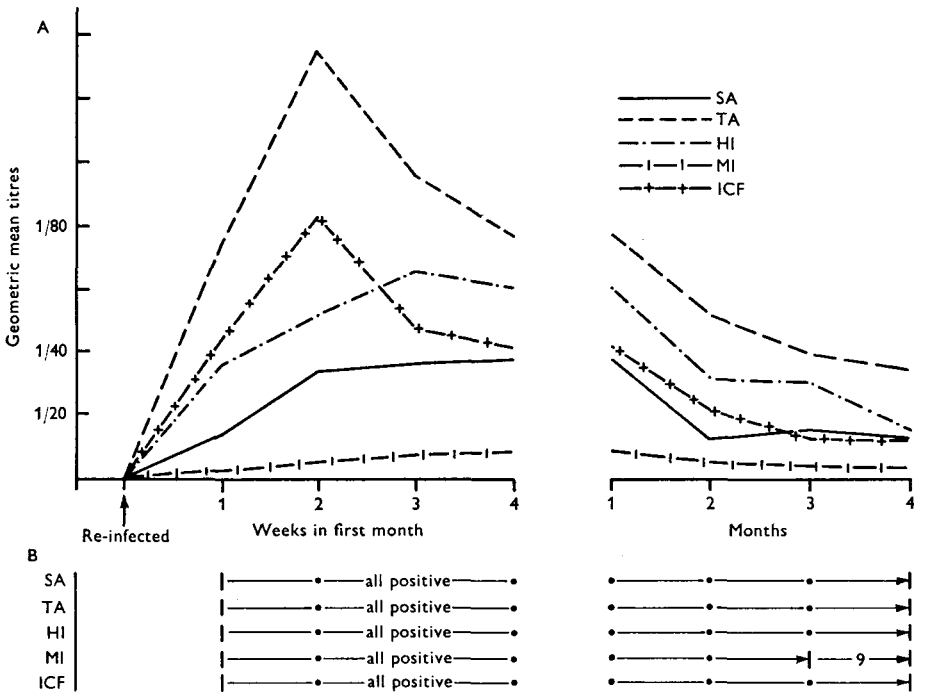

Fig. 7. A. Geometric mean titres of ten turkeys re-inoculated intratracheally with a culture of *M. gallisepticum*. B. The number of positive reactors by SA, TA, HI, MI and ICF tests.

Experiment 6

To study the serological response to re-infection in turkeys, fifteen of the twenty-one birds from Expt. 4 were housed separately in groups of ten and five respectively, 3 months after the initial infection. Each bird of the group of ten was re-inoculated intratracheally with 3 ml. of a culture of *M. gallisepticum* in T.I.M. containing 10^{10} viable organisms per ml. and the remaining group uninoculated. All fifteen turkeys were bled at the time of inoculation, at weekly intervals for 4 weeks, and then at monthly intervals for a further 3 months, and the sera examined.

At the time of re-infection the sera of all birds were negative by all tests for antibodies to *M. gallisepticum* and the pattern of serological response of the ten re-infected turkeys (Fig. 7) followed that obtained after a single infection (Fig. 5). However, after re-infection, the titres obtained by agglutination and HI tests were higher and, despite the anticomplementary nature of the sera, positive reactions were obtained by the ICF test. With this test a rapid rise and fall in titre was seen but, unlike the pattern with chicken sera (Figs. 4 and 6), peak titres were not as high as with the TA test. Although titres by the MI test were higher than after a single inoculation they never exceeded 1/16 and this was observed in only five of the ten birds. By the precipitation test one line was obtained with the sera of each of the ten re-inoculated turkeys 1 week after re-infection and three of the birds gave a positive precipitation reaction for as long as 3 weeks. All the birds gave positive reactions except by the MI and P tests up to 4 months, when the experiment ended. The five turkeys which were not re-inoculated remained negative to all tests.

Experiment 7

The serological response of chickens to immunization with *M. gallisepticum* was studied and compared with the response in rabbits.

An emulsion of *M. gallisepticum* grown in C.I.M. was prepared with Freund's complete adjuvant by mixing equal volumes of a culture of the organisms washed in phosphate-buffered saline pH 7·2 and concentrated 10 times, and adjuvant. Two ml. of the emulsion was inoculated subcutaneously into each of ten 18-month-old Brown Leghorn cockerels; 3 weeks later, each was given at weekly intervals six intravenous 1 ml. injections of the washed concentrated culture, which had been stored at $-27°$ C. The first two injections were the concentrated suspension as used previously, while the 3rd and 4th were twice as concentrated and the 5th and 6th were four and five times as concentrated respectively.

The birds were bled before inoculation, 1 week after the 6th intravenous inoculation and every 4 weeks for 2 months. Because at this stage the titre of the sera by the MI test was very low compared with that obtained for rabbit sera, one additional intravenous inoculation consisting of twice as many organisms as in the 6th intravenous inoculation was given to each chicken. The birds were bled 1 week later and then every 4 weeks for 5 months.

Three rabbits were immunized with the same organism and by the same methods as used for chickens except that the organism was grown in R.I.M. and no additional intravenous injections were given after the 6th. They were bled at the time of the initial inoculation and 1 week after the last intravenous injection and the sera examined.

The general pattern of response in chickens was similar to that following one or two intratracheal inoculations (Figs. 4 and 6). The pre-inoculation sera of the chickens were negative but 1 week after the 6th intravenous injection peak titres were observed (Fig. 8). Titres subsequently fell, but 1 week after the re-inoculation at the 9th week they peaked even higher than before and then fell again. The highest titres were obtained with the ICF test but they fell rapidly and were little higher than by the TA test at 9 weeks after the first series of inoculations. Following

re-inoculation at the 9th week titres by the ICF test again peaked higher than by the other test and did not again return to the level of the TA titres for 17 weeks. By all tests, titres were generally two or three dilutions higher than the corresponding ones following two intratracheal inoculations. Although titres were higher by the MI test than following intratracheal inoculations, they were rarely

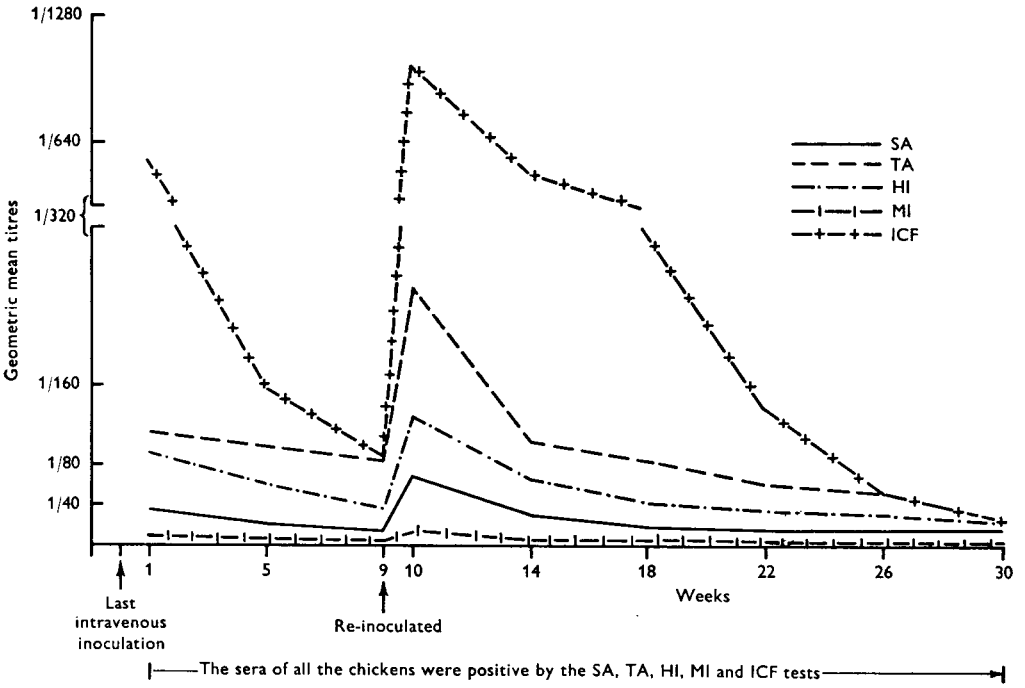

Fig. 8. Geometric mean titres of ten chickens hyperimmunized with a culture of *M. gallisepticum*.

Table 2. *Geometric mean peak titres of ten chickens, and geometric mean titres of three rabbits immunized with A* 514 *strain of* M. gallisepticum

	Test				
	SA	TA	HI	ICF	MI
Chicken	1/70	1/260	1/130	1/1024	1/14
Rabbit	1/127	1/640	1/403	Not done	1/812

higher than 1/32 and then only for the serum of one bird. By the precipitation test the sera were initially positive with three or four lines, but these gradually diminished in number and after 9 weeks the sera from two of the birds gave no precipitation at all. Following re-inoculation at the 9th week, all birds again showed between three and five lines of precipitation; and, although the number of lines declined, seven of the ten birds were still positive 21 weeks later.

The rabbit sera examined at 1 week after the 6th intravenous injection by the SA, TA, and HI tests showed titres higher by one or two dilutions than the chicken

sera even after the re-inoculation of the chickens at the 9th week (Table 2). In contrast to the low titres obtained with the chicken sera by the MI test the titres with rabbit sera were five or six dilutions higher. By the precipitation test three lines were seen with each serum.

DISCUSSION

Using the methods described in this paper for preparing and standardizing reactants and for performing the tests and using experimental birds housed in isolation and free from respiratory viruses and *M. gallisepticum*, all the pre-inoculation sera were negative. It was therefore assumed that a reaction by any of the tests, no matter how low the titre, was specific for *M. gallisepticum*.

The pattern of response after a single intratracheal inoculation of chickens showed that the highest titres were obtained with the ICF test, followed by the TA, HI, SA and MI tests. This is in keeping with the results of Boulanger & Rice (1953), who examined the sera of chickens for antibodies to Newcastle disease virus and found that higher titres were obtained by ICF than by the HI test.

With the ICF test a rapid rise was followed in 3 or 4 weeks by a rapid fall to below the titres of the TA test even when the chickens were inoculated on two occasions. However, after multiple inoculations the elevation of the ICF titre above that of the TA titre was maintained for a longer period. The titre of paired sera taken at an interval of about a week might be of value in indicating whether an infection or re-infection is recent. A disadvantage of the test as undertaken by us was that all chicken and turkey sera were found to be anticomplementary up to a dilution of 1/8 and 1/16 respectively, so that unless the amount of complement in the test was increased, thus reducing the peak titres, the test would be of little value in detecting low levels of antibody. It is interesting that in turkeys peak titres following two inoculations were not as high by the ICF test as by the TA test.

The titres obtained by the TA test were higher and persisted longer than those obtained by the HI and SA tests but the general pattern of response to single or double intratracheal inoculations in chicken and turkey sera and to multiple inoculations in chicken sera was similar by all three tests. This is in agreement with the results of Adler (1958), who found higher titres by the TA than by the SA test with turkey sera, and Baharsefat & Adler (1965), who obtained higher titres by TA than by HI with chicken and turkey sera taken soon after infection but the reverse with sera taken later. Hofstad (1957) and Rhoades *et al.* (1965), however, found titres by the HI and TA test to be similar for turkey sera but Hofstad observed positive reactions by the HI test to persist for a longer period. In contrast Jungherr *et al.* (1955) detected a larger number of reactors with chicken sera by the SA than by the HI or TA test.

Adler & DaMassa (1964) and Baharsefat & Adler (1965) found the antiglobulin test more sensitive than the TA or HI test.

It should be pointed out that the amount of haemagglutinating antigen used in the HI test will influence its sensitivity. Jungherr *et al.* (1955) used 2 HA units standardized to 100% end-point and Hofstad (1957) used 2 HA units, but the

end-point is not indicated. Baharsefat & Adler (1965) used the same technique, whereas we used 4 HA units standardized to 100% end-point. We found it essential to use this quantity of antigen in order to obviate non-specific inhibitors, and found it more effective than using receptor-destroying enzyme. Newnham (1964) also used 4 HA units for this purpose but no indication is given of the end-point in the standardization. In the standardization and in the test itself the 50% end-point should give greater accuracy but we found that 100% end-point was easier to determine when reading the test.

Although the SA test was found to be less sensitive than the TA or HI test, it has the advantage of being rapid and easily performed and therefore can be utilized as a routine flock test. The time interval between mixing the reactants and reading the test is influenced not only by the ambient temperature but also perhaps by the nature of the antigen, which in turn will be influenced by such factors as the strain of organism, and the method of culture, harvesting and standardization. Differences in these may account for the differences in the recommended time interval. For instance, Adler (1954) and Jerstad, Hamilton & Smith (1959) suggested that this period should be 5 and 8 min. respectively for turkey sera. These factors may also account for the unsatisfactory results in the test obtained by Jungherr *et al.* (1955) and Hofstad (1957), both with turkey sera.

The whole blood test was the least sensitive of the agglutination tests. Our technique closely followed that of Jungherr *et al.* (1955), using an equal volume of blood and stained antigen. Aftosmis, Tourtellotte & Jacobs (1960) drew attention to the importance of the ambient temperature and in our hands it never fell below 65° F. and we found no increase in the number of reactors by prolonging the time of the test beyond 2 min. for chickens and 3 min. for turkeys, when using the antigen prepared by ourselves or a commercially available one.* Despite the relatively smaller number of reactors and the relatively shorter period of their detection by this test it has several advantages. It is simple and easy to use, it is rapid and can be performed on the farm, and the birds need be handled only once. Dust on the slide may interfere with reading of the test and adequate precautions should be taken in a dusty environment.

With the MI test only very low titres were obtained even after two intratracheal inoculations of chickens and turkeys and after multiple inoculations of chickens. On the other hand sera from rabbits after multiple inoculations showed a high titre for MI antibodies. This would indicate that either only low-titre sera for the MI test can be prepared in chickens or turkeys, using our inoculum, or that a co-factor is necessary in the test. Unheated horse serum is an essential co-factor for the demonstration of fermentation-inhibiting antibodies in rabbit serum to *M. pneumoniae* and to *M. fermentans*, and to the Negroni agent, and unheated guinea-pig serum is an additional essential co-factor for the Negroni agent (Taylor-Robinson, Purcell, Wong & Chanock, 1966) .In our hands the addition of unheated horse and guinea-pig sera did not increase the MI titre of chicken, turkey and rabbit antiserum.

* Burroughs Wellcome.

https://doi.org/10.1017/S0022172400041115 Published online by Cambridge University Press

Precipitation lines were produced by only a small proportion of chickens and none of the turkeys after one intratracheal inoculation whereas they were observed in the sera of both species 1 week after two or more inoculations and for a few weeks afterwards.

In our experiments antibodies could be detected in all chickens and turkeys within 1 week of infection by the TA, SA and HI tests for chicken and turkey sera and by the ICF test for chicken sera. Peak titres were recorded by these tests between the first and second week. This is in contrast with the observations of Fahey & Crawley (1954), who infected day-old chickens by intranasal drop of an infected tissue suspension containing 'a PPLO and the virus previously described', probably *M. gallisepticum* and the Fahey–Crawley virus, and found that positive titres by the HI test first appeared at 9 days for some birds and by 29 days all were positive, and peak titres appeared after 10 weeks. Newnham (1964) infected chickens and turkeys with exudate from stock infected with the A 514 strain of *M. gallisepticum* and found that positive titres by the HI test were observed in the chickens after 3 weeks and in turkeys after 1 week and that peak titres were obtained at 6–9 weeks and 3–9 weeks respectively. Roberts (1964*a*, *b*) inoculated groups of chickens with broth culture of the S 6 strain of *M. gallisepticum* via the intranasal sinuses, the trachea, and posterior abdominal air sacs, and a group of turkey poults via the infra-orbital sinuses. In both chickens and turkeys maximum titres by the HI test were observed 4 weeks after infection.

The titres for chicken and turkey sera and the duration of positive reactions with turkey sera recorded by us have been less than those obtained by other workers. For instance, Fahey & Crawley (1954) observed peak titres by the HI test of 1/160 in chicken sera, and with turkey sera Hofstad (1957) and Crawley (1960) reported peak HI titres of 1/320 and 1/640 respectively and positive HI titres for 11–15 months. Newnham (1964), also using the HI test, observed peak titres with chicken and turkey sera of 1/640 to 1/5120, and Roberts (1964*a*, *b*) found peak titres by the HI test of 1/160 for both chickens and turkeys and positive reactions in most turkeys for at least 19 months.

The earlier appearance of positive reactions and peak titres in our experiments may be due to the large number of organisms in the initial inoculum. In addition it should be pointed out that the peak titres obtained with both chicken and turkey sera in our experiments were no greater than the titres observed by Newnham (1964) at the corresponding period after infection, but in Newnham's experiments the titres continued to rise for several weeks. The higher and later peak titres and the longer duration of a positive response seen by Fahey & Crawley (1954), Newnham (1964) and Roberts (1964*a*, *b*) are very probably associated with the virulence of the organisms in the inoculum. In our experiments the organism had possibly lost virulence by repeated culture in artificial medium and there was no evidence of disease in the infected chickens or turkeys, whereas Fahey & Crawley (1954) and Newnham (1964) observed symptoms and lesions, and the rise of HI antibody closely followed the development of clinical disease. Adler, Shifrine & Ortmayer (1962) infected turkeys by intranasal and intraperitoneal inoculation and examined the sera by SA, TA, and HI tests and concluded that absence of

lesions was usually reflected in absence of, or only low-titre antibody and that high titres of 1/80 and above would generally indicate disease rather than infection in which symptoms and macroscopic lesions were absent.

Roberts (1964*b*) examined the sera of eighty-six turkeys 19 weeks after infection, and noted that of sixty-three with positive titres by the HI test forty-four showed lesions, but all of twenty-three with no reactions to the test showed no lesions.

It is unlikely that the site of inoculation of our experimental birds was associated with the relatively low titres obtained. Intratracheal inoculation was chosen because there was less probability of wastage of organisms than following inoculation into the infra-orbital sinus or by nasal instillation as was practised by most other workers. Furthermore, Roberts (1964*a*) found that following the inoculation of three groups of chickens in the nasal sinus, trachea and posterior abdominal air sac respectively, the highest titre by the HI test was seen in many more of the birds inoculated via the trachea than by the other routes. Rhoades *et al.* (1965) inoculated three groups of six turkeys each with a culture of *M. gallisepticum* containing 10^7 organisms per ml. One group was inoculated intratracheally with 0·2 ml. per bird, another group with 0·4 ml. intranasally and the third via the right infra-orbital sinus with 1·0 ml. per bird. A fourth group was infected by exposure to these infected birds. Blood samples were taken weekly and examined by TA and HI tests. The initial antibody response was greatest in turkeys infected by the intratracheal and intrasinal routes but after 4 weeks differences were less obvious.

In our experiments higher titres were observed with chicken than with turkey sera and antibodies persisted for a longer time. Both chickens and turkeys were inoculated with approximately the same number of organisms, which means that the chickens received approximately 4–6 times as large a dose on a live weight basis. If the organisms multiplied in the infected host, however, this should be of little significance and it appears that, with our inoculum, turkeys did not respond as well as chickens. Newnham (1964) found no appreciable difference in the titre of the sera of chickens and turkeys by the HI test after infection with the A 514 strain of organism.

Re-infection of chickens by intratracheal inoculation 5 months after the initial infection, when antibodies could only be detected by the SA, TA and HI tests, and similar re-infection of turkeys 3 months after initial infection, when no antibodies could be detected, gave rise to positive reactions by all tests with titres higher than observed after the initial infection. Fahey & Crawley (1954) found that re-infection of chickens by the intranasal route, 77 days after initial infection, increased the titre of pooled sera examined by the HI test from 1/40 to 1/80. Adler *et al.* (1962) infected turkeys by the intranasal route and challenged them by intraperitoneal infection 7 weeks later, when titres were 1/10 or less and there was no clinical sign of disease. Little change in titre was observed when the sera were tested by SA, TA and HI tests. Roberts (1964*b*) re-infected a group of turkeys by inoculation into the infra-orbital sinus 19 weeks after the initial infection, when they showed no clinical signs of disease. He found that there was an antibody response by the HI

test in those turkeys which were negative at the time of re-infection but not in those showing positive titres (1/5 to 1/20) at that time.

It is difficult to explain the apparent lack of response found by these workers, especially in turkeys, to reinfection with *M. gallisepticum* when a positive titre already exists.

The MI test using sera prepared in rabbits may be a useful method for the classification of avian mycoplasma because the test can be performed without having to prepare and standardize antigen. However, it would be necessary to examine the specificity of the test.

Antisera to *M. gallisepticum* can be prepared in chickens which is comparable in titre to that produced in rabbits except for the MI titre.

SUMMARY

A comparison was undertaken of several serological tests in determining the response of chickens and turkeys experimentally infected with the A514 strain of *Mycoplasma gallisepticum*.

After a single intratracheal inoculation of chickens with a culture of the organism, the highest titres were obtained by the indirect complement fixation (ICF) test, followed by the tube agglutination (TA), haemagglutination inhibition (HI), slide agglutination (SA) and metabolic inhibition (MI) tests. By all these tests positive titres were observed within the first week and peak titres between the first and second weeks. At 5 months there was no positive reaction by the ICF test but most chickens gave positive readings by the TA, HI and SA tests for at least 14 months after infection, but turkey sera became negative by all tests after 3 months.

A disadvantage of the ICF test was that sera up to a dilution of 1/8 and 1/16 for chicken and turkey respectively were anticomplementary, and in turkeys this masked the ICF titre, which presumably was low following one intratracheal inoculation. Titres in turkeys with the TA, HI and SA tests followed the pattern seen with chickens and were generally lower than those found by other workers probably because of the avirulent nature of the inoculum used.

The WB test was the least sensitive of the agglutination tests but is useful as a flock test which can be undertaken on the farm.

The MI test gave the lowest titres of all and antibodies could be detected for only 4 months following one intratracheal inoculation. Even with serum prepared by multiple inoculations in chickens the titre was never higher than 1/32 compared with 1/1024 for serum similarly prepared in rabbits.

Precipitins were detected by the agar gel method in the sera of chickens and turkeys after two intratracheal inoculations but in only some of the chickens and none of the turkeys after one inoculation.

By all tests higher titres were observed with chicken than turkey sera and antibodies persisted for a longer time.

Re-infection of chickens when antibodies to the initial infection had become low, and of turkeys when antibodies were no longer detectable, gave rise to an anamnestic response with titres which were higher than before.

Antiserum to *M. gallisepticum* prepared in chickens is comparable with that prepared in rabbits except for low titres by the MI test.

We are indebted to the Agricultural Research Council for a grant towards the cost of this work.

REFERENCES

ADLER, H. E. (1954). A rapid slide agglutination test for the diagnosis of chronic respiratory disease in the field and laboratory infected chickens and turkeys—a preliminary report. *Proc. Am. vet. med. Ass. Ann. Meeting*, p. 346.

ADLER, H. E. (1958). A PPLO slide agglutination test for the detection of infectious sinusitis of turkeys. *Poult. Sci.* **37**, 1116.

ADLER, H. E. & DAMASSA, A. J. (1964). Enhancement of mycoplasma agglutination titres by use of anti-globulin. *Proc. Soc. exp. Biol. Med.* **116**, 608.

ADLER, H. E., SHIFRINE, M. & ORTMAYER, H. (1962). Interpretation of *Mycoplasma gallisepticum* serological tests for turkeys. *XIIth Wld's Poult. Congr.* p. 322.

AFTOSMIS, T. G., TOURTELLOTTE, M. E. & JACOBS, R. E. (1960). A sensitive whole blood test for *Mycoplasma gallisepticum*. *Avian Dis.* **4**, 485.

BAHARSEFAT, M. & ADLER, H. E. (1965). Comparison of tube agglutination, haemagglutination-inhibition (HI) and antiglobulin titres on sera of chickens and turkeys infected with *Mycoplasma gallisepticum*. *Avian Dis.* **9**, 460.

BOULANGER, P. & RICE, C. E. (1953). A study of complement fixation methods as applied to the demonstration of antibodies in birds. *Proc. Am. vet. med. Ass., Ann. Meeting*, p. 316.

CRAWLEY, J. F. (1960). Use of the hemagglutination-inhibition test in the control of chronic respiratory disease of chickens. *Ann. N.Y. Acad. Sci.* **79**, 562.

FAHEY, J. E. & CRAWLEY, J. F. (1954). Studies on chronic respiratory disease of chickens. IV. A haemagglutination-inhibition diagnostic test. *Can. J. comp. Med.* **18**, 264.

FEINBERG, J. G. (1956). Agar clarification. *Nature, Lond.* **178**, 1406.

HOFSTAD, M. S. (1957). A serological study of infectious sinusitis in turkeys. *Avian Dis.* **1**, 170.

HOFSTAD, M. S. & DOERR, L. (1956). A chicken meat infusion medium enriched with avian serum for cultivation of an avian PPLO, *Mycoplasma gallinarum*. *Cornell Vet.* **46**, 439.

JERSTAD, A. C., HAMILTON, C. M. & SMITH, J. E. (1959). Egg transmission of infectious sinusitis in naturally infected turkeys. *Avian Dis.* **3**, 28.

JUNGHERR, E. L., LUGINBUHL, R. E., TOURTELLOTTE, M. & BURR, W. E. (1955). Significance of serological testing for chronic respiratory disease. *Proc. Am. vet. med. Ass. Ann. Meeting*, p. 315.

LEACH, R. H. & BLAXLAND, J. D. (1966). The need for standardisation of serological techniques for the detection of *M. gallisepticum* infection in poultry. *Vet. Rec.* **79**, 308.

NEWNHAM, A. G. (1964). The haemagglutination-inhibition (HI) test and a study of its use in experimental avian respiratory mycoplasmosis. *Res. vet. Sci.* **5**, 245.

RHOADES, K. R., KELTON, W. H. & HEDDLESTON, K. L. (1965). Serological, pathologic and symptomatic aspects of mycoplasmosis of turkeys. *Can. J. comp. Med.* **29**, 169.

ROBERTS, D. H. (1964*a*). Experimental infection of chickens with *Mycoplasma gallisepticum* and subsequent re-isolation of the organism from the body tissues. *Vet. Rec.* **76**, 798.

ROBERTS, D. H. (1964*b*). Immunological aspects of infectious sinusitis in turkeys. *Vet. Rec.* **76**, 1200.

TAYLOR-ROBINSON, D., PURCELL, R. H., WONG, D. C. & CHANOCK, R. M. (1966). A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. *J. Hyg., Camb.* **64**, 91.