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# Detection of *Bordetella pertussis* antibodies in human sera by complement-fixation and immunofluorescence

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## SUMMARY

The complement-fixation test, as commonly used in the diagnosis of viral infections, was studied for its possible application to the diagnosis of whooping cough and the detection of antibody following pertussis vaccination. The results were compared with those obtained in parallel immunofluorescence tests. CFTs were performed on sera from 41 patients with whooping cough (*Bordetella pertussis* isolated), 125 vaccinated persons, and 618 controls; parallel tests by IF were made on sera from 24 cases of whooping cough, 36 vaccinated persons and 37 controls. Results of both tests correlated closely and showed that titres of diagnostic significance were seldom found in control sera. They also showed that, in patients suffering from whooping cough, antibody in a single specimen or a rise in antibody between paired sera was almost always demonstrated. Titres in general were lower in infants less than 6 months of age. IgG antibodies were involved in both tests. Although the number of sera tested was small both tests appear to be reliable as means of demonstrating the presence of antibody formed during the course of infection and after vaccination.

## INTRODUCTION

The use of the complement-fixation test (CFT) as a means of indicating current infection has been found valuable in the diagnosis of bacterial, viral and parasitic infections. It has been used in whooping cough since its original performance by Bordet & Gengou (1906, 1907) but not routinely in diagnostic laboratories in recent years. As complement fixation (CF) is used in many laboratories today for the diagnosis of viral infections it seemed worth while to investigate results in known cases of whooping cough and in persons whose vaccination history was known. In addition it was decided to examine a proportion of these sera by immunofluorescence (IFT) in order to compare results with those obtained in parallel CFTs, and to attempt to identify the class of immunoglobulin involved in the fixation of complement.

## MATERIALS AND METHODS

CFTs were performed on a total of 827 sera from 784 persons as follows:

(1) Forty-one patients with whooping cough from whom *Bordetella pertussis* was isolated;

(2) two groups of children in good health who had been vaccinated:

(i) Seventy-eight children under 5 years of age who had been given a primary vaccination course and a booster 18 months later (vaccine used not recorded). Sera were obtained at the following times after the booster injection: five sera at 4–5 months, 24 at 1 year, 23 at 2 years, 19 at 3 years, seven not known;

(ii) Forty-seven children under 2 years of age who were receiving a course of primary vaccination and booster with one of two different vaccines: 16 children were given vaccine A, which was Trivax containing aluminium hydroxide; 31 children were given vaccine B which was plain Trivax. Both vaccines were prepared by Wellcome Research Laboratories, Beckenham. During the course of vaccination random specimens were obtained according to availability. These might be either before or after primary vaccination or before or after the booster. It was not possible to obtain the whole series of specimens from any individual. A single specimen was obtained from 38 children, two specimens from eight and three from one child. Six and 32 sera were taken before and after vaccination respectively, and 8 and 11 before and after a booster respectively.

All sera in Group 2 (i) and (ii) were tested under code.

(3) Six hundred and eighteen persons aged 6 months to more than 80 years as controls: 147 were under investigation for hydatid disease; 34 for leptospiral infection; the rest were believed to be in good health.

IFTs were performed in parallel with CFTs on 24 sera from Group 1, 36 from Group 2(ii), and 37 from Group 3.

Six hundred and thirty-five specimens of sera received wet were stored either at 4° C. for periods up to 24 hr. or at −20° C. until the day they were tested. One hundred and ninety-two specimens received freeze-dried were stored at 4° C. until they were reconstituted in distilled water on the day of testing. All sera were heated at 56° C. for 30 min. immediately before the CFT was performed. All titres throughout this paper are expressed as reciprocals. Doubling dilutions of sera ranging from 10 to 640 were tested by CF. In IFTs doubling dilutions of 5–20 were tested initially, and further dilutions were tested when no end-point had been obtained.

### *Preparation of pertussis antigens*

*For complement-fixation tests*

Strains GL 353 (Type 1), BT 2 (Type 1, 3), 360 E (Type 1, 2), LN 16 (Type 1, 3; mainly 3), kindly supplied by Dr N. W. Preston of the University of Manchester, were used for the preparation of antigens by a method based on that described by Weichsel & Douglas (1937).

A 48-hour growth of *B. pertussis* on charcoal blood agar plates (85 mm. in diameter) was washed off each into 2 ml. sterile physiological saline. After shaking

vigorously for a few minutes, the suspension was heated for one hour at 60° C. It was then centrifuged at about 3000 rev./min. for 20 min. The slightly opaque supernatant fluid was used as an antigen. Sodium azide (0·08 % final concentration) was added as preservative. Antigens remained potent for at least 6 months when stored at 4° C.

*For immunofluorescence tests*

The suspension of killed *B. pertussis* organisms which had been prepared for agglutination tests on diagnostic antisera raised in rabbits, was used as antigen. It was made in 1963 from strains L92 and 18323K (Type 1, 2 and 4) and 3747 (Type 1, 2, 5 and 6) kindly supplied by Dr A. F. B. Standfast of the Lister Institute of Preventive Medicine, Elstree. Briefly, the suspension consisted of whole bacterial organisms which were grown on plates of Bordet Gengou agar for 3 days and then washed off into 0·25 % buffered formol saline containing glass beads. The suspension, with beads, was placed on a shaker overnight to break up any clumps of organisms. The suspension was then washed to remove the formalin. The organisms were finally resuspended in saline containing 1/10,000 merthiolate to a concentration equivalent to about $2 \times 10^{10}$ *Escherichia coli* organisms per ml., and were stored thereafter at 4° C.

*Serological tests*

*The complement-fixation test*

All CFTs were performed by the method described by Bradstreet & Taylor (1962). Three HD 50 of complement were used; tests were incubated overnight at 4° C. before the sensitized sheep cells were added. Antigens were standardized in chessboard titrations with individual sera from cases of whooping cough obtained during convalescence and from these the optimal dilution of each antigen for use in single line serum titrations was selected. At first, antigens prepared from all four strains were used to test sera but because results were very similar, subsequent tests were made with only two antigens, BT 2 and LN 16. Titres with each never varied by more than one dilution; the highest titre of a serum obtained with either antigen was the one recorded.

*The immunofluorescence test*

The IFT was performed as described by Edwards, Tannahill & Bradstreet (1970) but started with a dilution of 1/5 of the human serum and the stored agglutinating antigen was diluted 1/10. The conjugate was used at its optimal dilution, i.e. 1/10.

A swine antihuman IgG FITC conjugate prepared by Nordic Pharmaceuticals was used. This conjugate is prepared by immunizing swine with purified IgG from pooled normal human serum. Purified IgG is then extracted from the swine serum and conjugated with fluorescein. Although not absorbed with light chain components the reaction with other human immunoglobulins is not great.

A pool of human serum from known convalescent cases found strongly positive by IF, and a pool of human serum from sera previously tested by IF and found to be negative at a dilution of 1/5, were included as serum controls.

## RESULTS

The results of CFTs on the 618 control sera are presented in Table 1. Less than 7 % of these sera had titres > 10.

Results of CFTs on all sera from 41 known cases of whooping cough are shown in Table 2. This table also shows, where known, the age of the patients and the time since onset of the infection when the first specimens were taken. Two specimens of sera were obtained from 33 of the patients. The period after onset of disease when first specimens were collected was reported for 10 patients only, and ranged from 7 to 48 days. The time of collection of second specimens of sera (not shown in the table) was usually 2–3 weeks after the first. It can be seen that titres in general were lower in infants under 6 months of age than in older patients. Table 3 shows a comparison of titres obtained in the paired sera: of the 33 pairs, only 9, taken from patients who in Table 2 were numbered P 2, P 3, P 9, P 11, P 12, P 14, P 16, P 23 and P 25, did not in either specimen show a titre of antibody > 10; 7 of these pairs came from children of 4 months of age or less, and the remaining two from two children under 1 year. Where single specimens only were tested 5/8 had antibody titres > 10.

No table is presented to show the results on the sera from 78 children in Group 2 (i) because antibody was detected in only two specimens; one from a child 1 year after boosting had a titre of 10, and the other 2 years after boosting a titre of 20; all other sera were < 10.

Table 4 shows the results of CFTs on sera from children in Group 2 (ii): traces of antibody at a dilution of 1/10 were found in 2/6 sera from children before immunization with vaccine B; no antibodies were found in eight pre-booster specimens; all sera from children vaccinated with either two or three injections of vaccine A showed an antibody response which ranged from 10 to > 80, but there was a poor antibody response in sera from children immunized similarly with vaccine B for 7/18 had titres of < 10 and a further seven had a titre of 10 only; antibody was found in sera from 4/4 children following a booster injection with vaccine A, but in only 5/7 children after a booster injection with vaccine B. However, the two negatives were taken at 12 and 24 weeks and it appears that titres were lower after the 10th week.

A comparison of results obtained by CF and IF is shown in Table 5. Because in the CFT 83 control sera (13·4 %) gave titres of 10 (Table 1), and two pre-vaccinated

Table 1. *Complement-fixation tests on human control sera*

| | Serum titres | | | | | |
|---|---|---|---|---|---|---|
| Age (years) | < 10 | 10 | 20 | 40 | 80 | Total |
| 0–5 | 103 | 20 | 9 | — | 1 | 133 |
| 6–15 | 25 | 14 | 6 | — | — | 45 |
| 16–40 | 278 | 34 | 15 | 6 | — | 333 |
| > 40 | 62 | 5 | 3 | — | — | 70 |
| Not known | 26 | 10 | — | 1 | — | 37 |
| Total | 494 | 83 | 33 | 7 | 1 | 618 |
| % | 80 | 13·4 | 5·4 | 1·1 | 0·1 | 100 |

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Table 2. *Results of CF tests on sera from known cases of whooping cough*

| Patient no. P | Age Months | Days since onset | Paired sera 1st specimen | Paired sera 2nd specimen | Single specimen |
|---|---|---|---|---|---|
| 1 | 1 | — | 10 | 20 | — |
| 2 | 1 | — | < 10 | < 10 | — |
| 3 | 2 | — | < 10 | < 10 | — |
| 4 | 2 | — | — | — | 10 |
| 5 | 2 | — | — | — | 20 |
| 6 | 2 | — | < 10 | 20 | — |
| 7 | 3 | — | 10 | 80 | — |
| 8 | 3 | — | < 10 | 40 | — |
| 9 | 3 | — | < 10 | < 10 | — |
| 10 | 3 | — | < 10 | 20 | — |
| 11 | 3 | — | < 10 | 10 | — |
| 12 | 3 | — | < 10 | 10 | — |
| 13 | 3 | — | — | — | < 10 |
| 14 | 4 | 14 | < 10 | < 10 | — |
| 15 | 4 | 42 | — | — | 40 |
| 16 | 4 | — | < 10 | < 10 | — |
| 17 | 4 | — | < 10 | 20 | — |
| 18 | 6 | — | 10 | 80 | — |
| 19 | 6 | — | 10 | 20 | — |
| 20 | 8 | — | — | — | < 10 |
| 21 | 9 | — | 10 | 40 | — |
| 22 | 10 | 31 | — | — | 80 |
| 23 | 10 | — | < 10 | 10 | — |
| 24 | 11 | — | 40 | 80 | — |
| 25 | 11 | — | < 10 | 10 | — |
| | Years | | | | |
| 26 | 1 | 48 | 40 | 160 | — |
| 27 | 1 | — | 40 | 40 | — |
| 28 | 1 | 28 | — | — | 20 |
| 29 | 1 | — | 40 | 320 | — |
| 30 | 2 | — | 160 | 160 | — |
| 31 | 2 | — | < 10 | 80 | — |
| 32 | 2 | — | < 10 | 40 | — |
| 33 | 3 | 11 | < 10 | 80 | — |
| 34 | 3 | 10 | 80 | 40 | — |
| 35 | 3 | — | < 10 | 40 | — |
| 36 | 3 | — | 40 | 40 | — |
| 37 | 6 | 7 | 10 | 80 | — |
| 38 | 11 | — | < 10 | 40 | — |
| 39 | 11 | — | 10 | 40 | — |
| 40 | Adult | 21 | — | — | 40 |
| 41 | Not known | 14 | 80 | 160 | — |

Table 3. *Comparison of titres in paired sera from* 33 *patients*

| | |
|---|---|
| ⩾ 4-fold rise | 16 |
| 2-fold rise | 4 |
| No change: > 10 in both | 3 |
| Fall: > 10 in both | 1 |
| No change: ⩽ 10 in both | 9 |
| Total | 33 |

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Table 4. *Results of CFTs on sera from children during their primary course of injections*

| Serum no. | Vaccine | No. of injections | Pre-vaccination | Post-vaccination CF titre | Weeks* | Pre-boost titre | Months* | Post-boost titre | Weeks* |
|---|---|---|---|---|---|---|---|---|---|
| 1*a*, 1*b* | A | 2 | — | 20 | 3 | < 10 | 8 | — | — |
| 2 | A | 2 | — | ⩾ 80 | 4 | — | — | — | — |
| 3*a*, 3*b* | A | 2 | — | 20 | 8 | < 10 | 12 | — | — |
| 4*a*, 4*b* | A | 2+Bo | — | 10 | 16 | — | — | 40 | 5 |
| 5 | A | 3 | — | ⩾ 40 | 4 | — | — | — | — |
| 6*a*, 6*b* | A | 3+Bo | — | ⩾ 40 | 4 | — | — | 40 | 7 |
| 7 | A | 3 | — | 40 | 4 | — | — | — | — |
| 8 | A | 3 | — | 10 | 6 | — | — | — | — |
| 9 | A | 3 | — | 80 | 6 | — | — | — | — |
| 10 | A | 3 | — | 20 | 8 | — | — | — | — |
| 11 | A | 3 | — | 20 | 9 | — | — | — | — |
| 12 | A | 3 | — | 20 | 9 | — | — | — | — |
| 13*a*, 13*b* | A | 3+Bo | — | 40 | 9 | — | — | 40 | 6 |
| 14 | A | 3 | — | 10 | 15 | — | — | — | — |
| 15 | A | 3+Bo | — | — | — | — | — | 10 | 10 |
| 16 | A | 3 | < 10 | — | — | — | — | — | — |
| 17*a*, 17*b* | B | 2 | tr 10 | < 10 | 4 | — | — | — | — |
| 18*a*, 18*b* | B | 2+Bo | — | < 10 | 5 | < 10 | 11 | — | — |
| 19 | B | 2 | — | 10 | 9 | — | — | — | — |
| 20 | B | 2 | — | tr 10 | 10 | — | — | — | — |
| 21*a*, 21*b*, 21*c* | B | 2+Bo | < 10 | < 10 | 12 | — | — | < 10 | 12 |
| 22*a*, 22*b* | B | 2+Bo | — | < 10 | 14 | < 10 | 11 | — | — |
| 23 | B | 3 | — | 20 | 4 | — | — | — | — |
| 24 | B | 3 | — | 10 | 4 | — | — | — | — |
| 25 | B | 3 | — | 40 | 4 | — | — | — | — |
| 26 | B | 3 | — | < 10 | 5 | — | — | — | — |
| 27 | B | 3 | — | 10 | 5 | — | — | — | — |
| 28 | B | 3 | — | < 10 | 5 | — | — | — | — |
| 29 | B | 3 | — | 10 | 6 | — | — | — | — |
| 30 | B | 3 | — | 10 | 6 | — | — | — | — |
| 31 | B | 3 | — | 10 | 7 | — | — | — | — |
| 32 | B | 3 | — | tr 10 | 8 | — | — | — | — |
| 33 | B | 3 | — | < 10 | 13 | — | — | — | — |
| 34 | B | 3 | — | 80 | 13 | — | — | — | — |
| 35 | B | 3 | — | — | — | < 10 | 7 | — | — |
| 36 | B | 3 | — | — | — | < 10 | 8 | — | — |
| 37 | B | 3 | — | — | — | < 10 | 9 | — | — |
| 38 | B | 3 | — | — | — | < 10 | 10 | — | — |
| 39 | B | 3+Bo | — | — | — | — | — | 80 | 4 |
| 40 | B | 3+Bo | — | — | — | — | — | 20 | 4 |
| 41 | B | 3+Bo | — | — | — | — | — | 10 | 5 |
| 42 | B | 3+Bo | — | — | — | — | — | 20 | 11 |
| 43 | B | 3+Bo | — | — | — | — | — | 20 | 18 |
| 44 | B | 3+Bo | — | — | — | — | — | < 10 | 24 |
| 45 | B | 3 | < 10 | — | — | — | — | — | — |
| 46 | B | 3 | < 10 | — | — | — | — | — | — |
| 47 | B | 3 | tr 10 | — | — | — | — | — | — |

* = time since last injection; Bo = booster; tr = trace reading.

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children gave trace readings at 10 (Table 4), in this test a titre > 10 in a single specimen was accepted as one of diagnostic significance; by IF it was taken as five. Results were said to agree when, in a single specimen, both tests showed either no antibody or a significant titre as defined above and, in paired specimens, when both tests showed a rise in titre of four-fold or greater. In 88/97 tests there was agreement: 30 of these were positive and 58 were negative. Four sera which showed titres of 20 in IFs were negative in CFTs. Tests were repeated and CFTs remained negative even when performed in chessboard titrations with dilutions of antigen commencing at 1/2.

## DISCUSSION

In agreement with other reports our results show that CF antibodies to *B. pertussis* are found less frequently in infections which occur during the first months of life (a Combined Scottish Study, 1970) and that they appeared in the majority of patients during or shortly after an infection (Weichsel & Douglas, 1937; Wilson & Miles, 1964). Thus, in our studies, at the time the second specimen of serum was taken 24 of all 33 patients and 18/20 patients who were of 6 months of age or more had antibody titres > 10, and among single specimens tested the 3 with titres ⩽ 10 were from patients under 1 year. Although sera from cases were not tested serially to study any change in titres during late convalescence and beyond, it is likely that antibodies would ultimately disappear since the majority of results with control sera were negative. Control sera with titres > 10 may have come from persons already immune whose antibodies had been boosted following recent contact with a case of whooping cough.

From the close correlation of results in parallel tests by CFT and IF (Table 5) it is shown that the antibodies involved in CF are of class IgG. The positive titres obtained by IF which were < 10 by CFT may reflect antigenic variation in the pertussis strains used in the preparation of the suspension since these were different from the ones used to prepare the CF antigen, or possibly the existence in the sera of different IgG components. The discrepancies do not appear to represent simply a difference in the sensitivities of the two tests because the levels of antibody titres in each, where significant, did not always run parallel.

The vaccine used to immunize the 78 children in Group 2(i) was different from those used for the children in Group 2(ii) and the titres of CF antibody present, if

Table 5. *Serum antibody titres in CF and IF tests performed in parallel*

| Source of sera | No. of sera | Agree | IF ⩾ 5 CF ⩽ 10 |
|---|---|---|---|
| Known positive | 24 | 18 | 6* |
| Vaccinated | 36 | 33 | 3† |
| Control | 37 | 37 | 0 |
| Total | 97 | 88 | 9 |
| % | 100 | 90·7 | 9·3 |

* Two sera with an IF titre of 5, one of 10, and three of 20.
† Two sera with an IF titre of 5, and one of 20.

any, in their early post-booster sera were not known. It was not possible, therefore, to interpret with certainty the finding of negative results in the 76/78 sera tested by us. However, it was this unexpected observation, especially in sera taken only 4 to 5 months after the booster injection, that initiated the investigation on the sera from children in Group 2(ii). From this Group an attempt was made to find out if CF antibody was at any time provoked by vaccination and, if so, how long it remained present. Although the number of children tested was small and sera were never obtained from any single child throughout the course of injections, we, nevertheless, feel able to draw a few tentative conclusions from the limited findings observed.

Significant titres of CF antibody are not detectable before vaccination; they are provoked to a greater or lesser extent by different vaccines, and 2 injections with a potent vaccine may provoke more antibody than three with a less potent one; CF antibodies are not detectable 7 months, or possibly sooner, after the primary course of vaccination and they reappear following a booster; lastly, the time of disappearance of antibodies after the booster injection is not known, but it may be as early as 4 to 5 months if the negative results obtained in the 78 children in Group 2(i) represents a fall in titre. These conclusions must, of course, be subject to confirmation by serial investigations on a larger number of children.

The manufacturers of vaccines A and B used in these studies stated in a personal communication that 'in six mouse protection tests the best estimates of potency was that vaccine A had a potency of 12 protective units per dose of vaccine and vaccine B had four'. This assessment correlates well with the titres of CF antibodies found in the children in Group 2(ii) given the two vaccines.

In general, antibodies of class IgM are known to agglutinate corresponding antigens more readily than those of IgG. The agglutination reaction was the test used in a series of investigations made by the Whooping Cough Immunization Committee of the Medical Research Council (Report, 1959) on the prophylactic value of vaccines and their assessment by a laboratory test. They showed that sera from mice immunized with an antigenic extract (Pillemer, Blum & Lepow, 1954) contained low titres of agglutinating antibody compared with those immunized with some whole bacterial vaccines, although the Pillemer antigen and bacterial vaccines both showed good protection in children. From this it was concluded that agglutinin production in mice could not always be taken as evidence of protective activity of a vaccine in children. Since then the assessment of protective activity of a vaccine in the laboratory has been made by means of the mouse protection test. If the Pillemer antigen – an antigenic extract – provoked mainly IgG antibodies these would agglutinate bacterial suspensions less efficiently than IgM, and this fact may account for the poor response found in mice which were vaccinated with Pillemer antigen. A CFT performed in these circumstances might have detected such IgG antibodies in mice as efficiently as the agglutination reaction detected IgM antibodies formed after vaccination with whole bacterial organisms. If it can be shown that CF titres in mice after vaccination correlate with protection in children, as CF titres in children appear in these studies to correlate with protection in mice, then it might be possible to use this test routinely for assessing the

potency of a vaccine in place of the mouse protection test which is more laborious and costly. Investigations are in progress to study this point.

Agglutination reactions for diagnosis were not made in this study because in our experience smooth stable killed suspensions can be difficult to prepare, and they may not be as sensitive in tests with human sera as suspensions of living organisms (Evans & Maitland, 1939). The extract used in the CFT reported here is easy to prepare, and it remains potent and free from anticomplementary activity when stored at 4° C. for long periods. The use of the CFT routinely in cases of suspected whooping cough in which an organism has not been isolated will provide a reliable indication in retrospect of infection due to *B. pertussis*, particularly in patients over 6 months of age. The use of IF as an alternative test would appear to be equally satisfactory.

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