Detection of *Chlamydia psittaci* by Immunofluorescence

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A direct fluorescent antibody (FA) test was developed to detect *Chlamydia psittaci* in dual impressions from specimen-inoculated mice. Technical procedures for the test were compared. *C. psittaci* was found in mice after infection as early by the FA technique as it was by cytochemical staining methods usually used. The lymphogranuloma venereum organism was also stained by conjugated antibody to *C. psittaci*. A distinctive advantage of the described FA test is that organisms are identified immunologically as members of the genus *Chlamydia* simultaneously with their detection.

The usual method for isolating *Chlamydia psittaci* from specimens is by inoculating mice or embryonated chicken eggs (15). To reveal and identify the organism, tissue impressions from these indicator hosts are then prepared and stained, generally by the techniques of Macchiavello (14) or Gimenez (10). Organisms of the genus *Chlamydia* present are recognized by morphological and tinctorial characteristics. More certain identification is desirable, however, because the appearance of these organisms is mimicked by coccii, *Mycoplasma synoviae*, and other microbes. Although positive identification may be accomplished by the complement fixation (CF) test or the toxin neutralization test with sera known to contain psittacosis antibodies, these tests require amounts of the agent often obtainable only by repeated passaging. Identification by infectivity neutralization tests is possible but also is too time consuming for routine use in a diagnostic laboratory.

It appeared that the direct fluorescent-antibody (FA) technique, if applied to tissue impressions from specimen-inoculated mice, should permit rapid immunological identification of the psittacosis agent. This possibility seemed especially likely because the technique has already been successfully applied to *C. psittaci* in tissue culture (3, 11, 17, 20). However, only limited success has been reported for detection of the organism in infected animals by FA staining. Donaldson et al. (8) obtained disappointing results with psittacosis-infected turkeys. Bates et al. (1) were able to demonstrate *C. psittaci* in no more than 80% of turkeys and 90% of mice experimentally infected; occasionally, spleen impressions from uninoculated mice were positively stained by normal control conjugate.

Application of the immunofluorescence method to *C. trachomatis*, an organism closely related to *C. psittaci*, has been both extensive and useful. For example, the technique was found by Nichols et al. (18) to be the diagnostic method of choice for conjunctival specimens. Nichols et al. (18) showed in an extensive epidemiological study of trachoma that the FA technique was practical to use in a field laboratory; the method yielded data more valuable epidemiologically than those obtained by clinical observations only. Hanna (12) reported that trauma resulting from scraping conjunctivas of chronically infected persons enhanced the prevalence of FA-positive trachomal inclusions in second scrapings collected after 1 to 7 days. The FA technique was utilized by Dawson et al. (7) to show that drug regimens producing clinical improvement in trachoma infections nevertheless failed to eliminate the organism from the conjunctiva. Bell and Fraser (2) stained *C. trachomatis* in conjunctival smears from experimentally infected monkeys with fluorescein-conjugated lymphogranuloma venereum (LGV) antiserum to demonstrate susceptibility to the infection.

Encouraged by the proven usefulness of the immunofluorescence method for trachoma, we undertook development of a direct FA test for identifying *C. psittaci* in brain impressions
from specimen-inoculated mice.

**MATERIALS AND METHODS**

**Organisms.** Table 1 lists the sources and passage histories of the *C. psittaci* strains used. The LGV organism employed was the JH strain, obtained from the American Type Culture Collection. Two strains of Colorado tick fever virus and a strain each of rabies virus and *Escherichia coli*, used in one of the tests for specificity of staining, were supplied by Center for Disease Control laboratories.

**Antiserum production.** To propagate psittacosis organisms for immunization, we grew Vero cell monolayers at 37 C in 32-oz prescription bottles with Eagle medium (9) containing 50 μg of streptomycin sulfate per milliliter and 10% fetal bovine serum. We inoculated the monolayers by replacing the growth medium with 10 ml of a 10% (w/v) homogenate prepared in maintenance medium from a portion of mouse brains in which strain 642 of *C. psittaci* was originally isolated. The maintenance medium had the same composition as the growth medium except for a reduction in serum concentration to 2%. After holding the inoculated monolayers at 37 C for 2 hr, we added 40 ml of maintenance medium to each. Medium was replaced 4 and 6 days after inoculation, and the final collection was made on day 11 when approximately 70% of the cells showed cytopathic effects. For passage of strain 642, the 4-, 6-, and 11-day medium was pooled to inoculate other monolayers in the manner described for inoculation of the mouse brain homogenate.

The pooled 4-, 6-, and 11-day medium from the fifth serial passage of strain 642 on Vero cells contained 10⁻³⁴ intracranial (ic) LD₅₀ per ml when titrated in 3-week-old mice. This pool was clarified by centrifugation at 1,000 × g for 10 min. The psittacosis organisms were pelleted from the clarified medium by centrifugation at 12,000 × g for 45 min, washed twice in phosphate buffered saline (PBS), pH 7.2, and were resuspended in PBS one-twentieth of the original volume.

Weekly, for 7 weeks, rabbits each received 2 ml of the antigen suspension divided equally for intravenous, intramuscular, intraperitoneal, and foot pad inoculation. The animals were bled 10 days after the last inoculation, and the pooled sera were stored at –35 C until fractionation. The CF titer of the serum pool, performed by the Laboratory Branch CF microtiter method (5), was 2,048.

After passing the LGV organism five times on Vero cell monolayers, we used it to produce antiserum by the procedure described for production of psittacosis antiserum.

To obtain sera for control conjugates, medium from uninfected Vero cell monolayers was collected and processed for rabbit inoculation in the same way as inoculum from the infected monolayers. The inoculation schedule followed was that used for production of psittacosis antiserum.

**Preparation of FA conjugates.** Fractionation was performed at 4 C. Globulin was precipitated from the immune rabbit sera by addition, in drops, of an equal volume of 80%-saturated (NH₄)₂ SO₄ solution. The precipitated globulin was collected by centrifugation, dissolved in distilled water, and again precipitated and dissolved as described. The (NH₄)₂ SO₄ was then removed by dialysis of the globulin solution against PBS, pH 7.2.

The dialyzed solution, adjusted to a protein concentration of 1%, was divided into two samples for conjugation (13) with fluorescein isothiocyanate (BBL). Conjugate A was prepared by using a dye-to-protein ratio of 1:30 and conjugate B by using a ratio of 1:100. Conjugates were freed of unreacted fluorescent material by dialysis at 4 C against PBS; after dialysis each ml of conjugate contained 5 mg of protein, consisting of 61% gamma globulin, 32% alpha and beta globulin, and 7% albumin. Conjugate A contained 15 μg of fluorescein per mg of protein and conjugate B contained 8 μg per mg. Control conjugates having comparable fluorescent and protein concentrations were prepared in the same manner from sera of the rabbits inoculated with processed harvests from uninfected Vero cell monolayers.

**Processing of brain impressions.** For brain impressions, the skull caps of mice were removed to leave the brains in situ with meninges intact. Glass slides were then touched to the exposed meninges. The resulting impressions were allowed to dry for 30 min at 25 C (room temperature) before fixation.

Unless stated otherwise, impressions were fixed with acetone for 1 hr at –20 C, exposed for 1 hr at 37 C to conjugate diluted in PBS containing 20% normal mouse brain homogenate, and finally washed for 20 min in gently stirred PBS. The washed impressions were dipped briefly in distilled water, allowed to dry at 25 C, and mounted with a coverslip and mounting solution, pH 7.2, consisting of 90% glycerol and 10% PBS. The mounted impressions were examined with a Zeiss RA microscope equipped with darkfield condenser and HBO 200 mercury vapor lamp. The most satisfactory filter combination was primary filters BG 38 and UG1 together with secondary filters no. 65 and 41.

Impressions were coded so that the microbiologist was unaware which were from uninfected mice, stained with control conjugate, or processed by mod-

| C. psittaci strain | Specimen          | Strain passage level* |
|-------------------|-------------------|-----------------------|
| RA 641            | Parrot, Washington state | 4                     |
| RA 647            | Parrot, Washington state | 1                     |
| RA 651            | Parakeet, Illinois  | 1                     |
| RA 849            | Macaw, Peru        | 3                     |
| RA 1791           | Parakeet, New Jersey | 1                     |
| RA 642            | Parrot, Washington state | 1                     |

* Number of passages before organisms were inoculated into mice for FA staining. Passages were by ic inoculation of 3-week-old mice.
ifications of the routine procedure.

RESULTS

Appearance of conjugate-treated impressions. The appearance of psittacosis-infected cells in mouse brain impressions exposed to immune conjugate is shown in Fig. 1. The impression was made from a 26-day-old mouse 5 days after inoculation with strain 849; the mouse appeared sick for 2 days before sacrifice. Small fluorescent granules, although usually discrete, were so numerous in occasional cells that almost the entire cytoplasm appeared to fluoresce. Often, one microscopic field contained several infected cells whereas an adjacent field held none. However, they usually occurred in sufficient numbers in positive impressions to be found within a few seconds.

LGV-infected cells and psittacosis-infected cells exposed to psittacosis immune conjugate were indistinguishable in appearance.

Staining specificity was established by using several controls. Immune conjugate dilutions that stained the psittacosis organism in brain impressions from infected mice did not produce staining in impressions from uninfected mice or from mice infected with rabies virus, two strains of Colorado tick fever virus, or one strain of E. coli. The absence of staining in the impressions from mice infected with these organisms indicates that staining in psittacosis-infected brain impressions is not attributable to host response to infection per se. Control conjugate did not produce staining in brain impressions from psittacosis-infected mice. Staining of the psittacosis organism in impressions from infected mice by immune conjugate was inhibited by unlabeled rabbit antisera to C. psittaci or the LGV organism, but not by normal rabbit serum. Staining was inhibited also by dilution of immune conjugate in PBS containing 20% homogenized brain from psittacosis-infected mice in place of the usual 20% normal mouse brain.

Technical procedures for FA staining. Temperatures and times for fixation were compared by immersing brain impressions in acetone at 25, 4, and -20°C for periods of 10, 30, or 60 min. Other impressions were heat fixed by one rapid passage through a flame. Staining by immune and control conjugate was graded on a scale from 4 (maximal fluorescence) to 0.

Non-specific staining by control conjugate appeared as diffuse, non-granular fluorescence. All impressions fixed at 25°C and many fixed at 4°C showed non-specific staining, having an intensity of at least 3. However, impressions fixed at -20°C or by heat showed non-specific staining no greater than 1 in intensity after staining. Specific staining in impressions fixed at -20°C for 60 min was at least as intense as that following any other time-temperature combination, and cells fixed by this treatment were not lost from the glass slide while being washed in PBS after staining. Therefore, all impressions henceforth were fixed by this procedure.

Fixed impressions were exposed to immune conjugate at 25°C or 37°C for 5, 10, 30, or 60 min. As shown in Table 2, specific staining attained the same maximum intensity after 30 min at either temperature.

Impressions that were washed for 5, 10, 20, or 30 min, in gently stirred or unstirred PBS after exposure to conjugate, appeared to retain

TABLE 2. Fluorescence of Chlamydia psittaci in infected mouse brain impressions stained at 25 or 37°C for various time periods

| Staining Concentration | Staining temp | FA conjugate dilution |
|------------------------|---------------|----------------------|
|                        | (C)           | 1:8            | 1:16 | 1:32 | 1:64 |
| 5                      | 25            | 2a              | 0    | 0    | 0    |
| 10                     | 25            | 3               | 3    | 1    | 0    |
| 10                     | 37            | 4               | 4    | 3    | 2    |
| 30                     | 25            | 3               | 2    | 0    | 0    |
| 30                     | 37            | 4               | 3    | 2    | 1    |
| 60                     | 25            | 4               | 4    | 3    | 2    |
| 60                     | 37            | 4               | 4    | 3    | 2    |

* Fluorescence was graded on a scale from 4 (maximal) to 0.
the same intensity of specific staining. Background fluorescence and that of cellular debris, however, became minimal after impressions were washed with gentle agitation for 20 min.

To compare methods for control of nonspecific staining, we diluted conjugate A in unsupplemented PBS and in PBS containing Evans blue (1:1,000), 20% homogenized normal mouse brain, or both. Nonspecific staining was not troublesome with dilutions >1:16 in plain PBS, >1:4 in PBS containing Evans blue, and >1:2 in PBS containing normal mouse brain either alone or with Evans blue. Conjugate A produced specific staining having an intensity of 4 when diluted 1:8 with PBS containing 20% normal mouse brain, apparently the diluent of choice. Conjugate B, containing the same protein concentration as conjugate A but half the fluorescein, produced specific staining with an intensity of 4 only at dilutions of 1:4 or less.

The stability of fluorescence in impressions after staining was greater upon storage at −35 C than at 4 or 25 C (Table 3). Impressions held at −35 C for the longest period tested (6 weeks), and before exposure to conjugate, were as satisfactory as freshly stained impressions.

Detection of C. psittaci in infected mouse brain. Groups of mice were infected by IC inoculation of the first five strains of C. psittaci that are listed in Table 1 and with the mouse passage levels indicated. One mouse from each group was sacrificed daily, and three brain impressions were prepared. Gimenez stain was applied to one impression, Macchiavello stain to another, and FA conjugate to the impression made last. Five mice were then inoculated IC with 0.03 ml of 10% suspensions of the same brains used for impressions.

A brain positive for psittacosis by any stain was invariably found to be positive by all (Table 4). Brains of inoculated mice always were infectious 2 days earlier than the psittacosis organism could be demonstrated by staining and at least a day earlier than the mice appeared sick.

DISCUSSION

The FA technique, of proven value for the routine diagnosis of many other microbial infections, also appears to be applicable to infections with C. psittaci. Results of this study suggest that the described test is genus specific, not species specific. The LGV organism, now classified as a member of the species C. trachomatis (21), was readily stained by psittacosis immune conjugate, and staining of C. psittaci was inhibited by unconjugated antisera against the LGV agent. These results are in accordance with those of Ross and Borman (20) who reported that a direct immunofluorescent technique was genus specific with cell cultures infected by the meningopneumonitis agent. Nevertheless, a species-specific FA test may be attainable in view of the success by Wang and Grayston (22) in differentiating eight immunological types of C. trachomatis by an indirect FA method. Perhaps the subspecies specificity may be attributed, at least in part, to production of highly specific antisera in mice by a short immunization schedule. An FA test for rapid identification of individual species belonging to the genus Chlamydia could prove helpful in some instances; fortunately, consideration of the source of positive specimens often provisionally indicates the species present.

The psittacosis organism was demonstrable in brain impressions from mice as early after infection by the staining techniques of Macchiavello or Gimenez as by FA staining. However, only the immunologically specific FA technique unequivocally identified the agent as a member of the genus Chlamydia. Because the psittacosis organism also may be identified by a CF test with antisera known to be specific

| Table 3. Fluorescence stability at various storage temperatures of FA-stained impressions from infected mouse brain |
| --- |
| Storage | Storage day |
| temp (C) | 0 | 1 | 2 | 4 | 7 | 14 | 28 | 65 |
| 25 | 4* | 4 | 2 | 1 | 0 | 0 | 0 | 0 |
| 4 | 4 | 4 | 4 | 2 | 2 | 2 | 2 | 2 |
| −35 | 4 | 4 | 4 | 4 | 4 | 4 | 2 | 2 |

* Fluorescence was graded on a scale from 4 (maximal) to 0.

| Table 4. Detection of Chlamydia psittaci in brains of inoculated mice |
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| C. psittaci strain | Day organism was first demonstrated |
| | Macchiavello stain | Gimenez stain | FA stain | Mouse infectivity |
| 849 | 4 | 4 | 4 | 2 |
| 641 | 3 | 3 | 3 | 1 |
| 1791 | 4 | 4 | 4 | 2 |
| 647 | 3 | 3 | 3 | 1 |
| 651 | 3 | 3 | 3 | 1 |
for C. psittaci, we attempted identification by using as antigen FA-positive mouse brain (unpublished data). The CF tests were negative; these results probably reflected an insufficient concentration of CF antigen in the infected brains.

In the most widely used application of the FA technique to diagnostic virology, the examination of rabies specimens, inclusion of a specificity (blocking) control with every specimen tested is recommended (6). Likewise, it would be prudent to establish staining specificity in the same way with each psittacosis specimen. We found staining to be effectively inhibited by diluting immune conjugate in PBS that contained 20% homogenized brain from mice dying of infection with C. psittaci.

Diagnosis of psittacosis in birds by the FA test would require even less time than the FA procedure described with mice if the test could be applied to impressions prepared directly from the bird organs. Because C. psittaci may be detected (21) in impressions from some infected birds by Macchiavello staining, definite identification should be possible by applying the FA test to such impressions. This possibility is currently being examined.

As emphasized by Carski (4), the usefulness and limitations of an FA diagnostic procedure may be established only through wide experience. The FA technique for psittacosis appears to merit such evaluation.

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