SPECIFIC STAINING OF VARIOUS BACTERIA WITH A SINGLE FLUORESCENT ANTIGLOBULIN

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Coons and Kaplan (1950) developed a rapid, sensitive, and specific fluorescent antibody tech-
ique for staining viruses in tissue sections. They used a direct staining procedure in which a spe-
cific fluorescent antibody preparation is applied directly to the cell being studied. In the indirect
procedure (Weller and Coons, 1954), the virus is first reacted with a specific nonfluorescent anti-
serum prepared in the rabbit and then reacted with fluorescent antiglobulin prepared against
rabbit globulin. The direct staining procedure was adapted to the staining of bacteria by Moody
et al. (1956) who used fluorescein-labeled antiserum to identify individual cells of Malleomyces
pseudomallei on glass slides. Fluorescent antibody preparations have been used to identify Hem-
ophilus pertussis (de Repentigny and Frapprier, 1956), Malleomyces pseudomallei (Thomason et al.,
1956), and Salmonella species (Thomason et al., 1957).

This paper is an extension of a preliminary re-
port (Carter and Leise, 1957) and presents the appli-
cation of the indirect staining techniques to the identification of Brucella suis strain PSIIIIK,
Pasteurella tularensis Schu, Vibrio comma strain V8, and Pasteurella pestis strain A1122, using a
single fluorescent rabbit antiglobulin. Data pertaining to the application of the direct staining
technique to the above organisms are also pre-
sented.

MATERIALS AND METHODS

All antigens, except P. pestis, used for anti-
serum production were prepared by growing the
cells on agar plates at 37 C, and harvesting the
growth in formalized (0.3 per cent) saline. A
living suspension of P. pestis was used for anti-
serum production. The suspensions contained
approximately 1 x 10^8 cells per ml.

Rabbits were injected intravenously 3 times a
week for 2 weeks with 0.5 ml of the prepared
bacterial suspensions. The rabbits were bled 7
days after the last injection. The P. pestis anti-
serum had a titer of 1:1280; the other antisera,
1:640. Two of the rabbits were subjected to an
additional immunizing series with B. suis and
P. pestis antigens and yielded antisera of titers
1:2580 and 1:5160, respectively. The \( \gamma \)-globulin
was extracted from serum using the method
described by Dubert et al. (1953), which involves
the precipitation of serum with methanol. This
globulin was then labeled with fluorescein iso-
cyanate by the method of Coons and Kaplan
(1950).

Rabbit antiglobulin was prepared by injecting
a goat 3 times a week for 4 weeks with 1 ml
amounts of increasing concentrations (40, 50,
60, and 70 mg) of normal rabbit \( \gamma \)-globulin. The
goose was bled 10 days after the last injection.
The globulin was then precipitated from this
antisera and labeled with fluorescein isocyanate.

The fluorescent staining of bacteria by the
indirect technique (figure 1) was accomplished
by preparing bacterial smears on glass slides and
then heat fixing after drying. Specific antisera,
present in the rabbit, undiluted or diluted 1:10
were added to the bacterial smears and allowed
to react for 10 to 15 min. The slide was then
washed in phosphate buffered (pH 8) saline
("dried" by shaking off the water droplets) and
a 1:5 dilution of the fluorescent rabbit antigu-
bulin added to the smear. After 10 min, the slide
was washed, "dried" by shaking, a drop of
phosphate buffered glycerol (pH 8) added and a
cover slip placed over the stained area. The stain
was then examined under the oil immersion
objective using a Leitz Ortholux microscope with
an ultraviolet light source. All experiments

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Direct Staining Technique
Bacteria + fluorescent immune globulin → fluorescent bacteria

Indirect Staining Technique
Bacteria + immune globulin → bacteria immune globulin complex
Bacteria immune globulin complex + fluorescent antiglobulin → fluorescent bacteria

Figure 1. Direct and indirect staining techniques

RESULTS
In all instances, results with the indirect technique were specific, for staining occurred only when the bacteria had been previously reacted with their specific antisera.

B. suis antisera cross-reacted with cells of P. tularensis when an antisera of 1:2580 titer was used. Diluting the antisera did not eliminate this cross-reaction. The cross-reaction was eliminated, however, by the use of a low titered (1:640) antisera for B. suis. Of two antisera for P. pestis, one with a titer of 1:5160 cross-reacted with Pasteurella pseudotuberculosis in both the undiluted and 1:10 dilution; the other with a titer of 1:1280 did not.

Both formalin-killed and living suspensions of B. suis, P. tularensis, and V. comma stained satisfactorily. However, a 24 to 48 hr living suspension of P. pestis was required to obtain maximum staining of the cells. When a formalin-killed 24 to 48 hr old culture or a 72-hr or older living suspension was used, some of the cells did not stain.

In staining an unknown slide, it is possible to wash the smear area after a negative reaction has been obtained, and to repeat the test with another antisera. This procedure was followed with B. suis, using successive applications of antisera to V. comma, P. pestis, P. tularensis, and B. suis. Each application of antisera was followed by the addition of fluorescent antiglobulin. Fluorescent cells resulted only when Brucella antisera and fluorescent antiglobulin were applied. The fluorescence was definite but somewhat weaker than that usually obtained by the standard procedures.

A similar result was obtained with the direct technique when cells of B. suis were exposed to fluorescent antisera for P. tularensis, washed, and then exposed to fluorescent antisera for B. suis.

Small numbers of B. suis (formalin-killed suspension) and P. pestis (24-hr culture) were detectable in pure and mixed culture by the indirect staining technique (table 1). When at least $6 \times 10^8$ cells were placed on a 225 mm² area, it was possible to detect the presence of fluorescent cells without much difficulty. However, when $6 \times 10^6$ cells or less were so placed the entire stained area had to be scanned in order to find fluorescent cells.

An attempt was made to collect and specifically stain bacteria on membrane filters. However, this could not be done because of the natural fluorescence of the filter, and the difficulty of passing the ultraviolet light through the filter³. B. suis was collected on a membrane filter, transferred to a glass slide by pressing the face of recent studies show that 12-hour old colonies of Bacillus anthracis can be stained with fluorescent antisera on black (non-fluorescing) Millipore membrane filters using a top lighting technique and can be observed with a low power dissecting microscope.
of the filter against the slide with a firm object, and then stained on the slide. Only the direct staining technique was used here, but the indirect procedure should work as well.

In all other experiments, the indirect staining procedure gave results similar to those obtained with the direct procedure.

**DISCUSSION**

The results obtained in these studies indicate that bacteria may be identified with a single fluorescent antiglobulin used in conjunction with specific nonfluorescent antisera. This indirect method of fluorescent staining has the advantage of requiring the preparation, testing, and storage of only one fluorescent antiglobulin which may be used with a number of specific microbial antisera for the identification of various bacterial species. This eliminates the necessity of preparing a fluorescent antiserum for each organism to be studied, as required by the direct staining procedure.

It has been usual in serological studies to use antisera of high antibody titer. Such antisera will produce cross reactions when tested with related organisms. However, by producing antisera of low titer, one apparently does not obtain antibodies to minor antigens; thus the annoyance of cross reactions is lessened. Noncross-reacting antisera are quite advantageous for identification purposes. In antigenic analysis studies, high titered antisera containing antibodies to all possible antigens would, of course, be most advantageous.

In the indirect technique, the specific antiserum was applied either undiluted or diluted 1:10. A higher dilution of the antiserum resulted in a decreased fluorescence after the fluorescent antiglobulin was applied. This decreased fluorescence seemed to depend upon both the dilution of the antiserum and upon the particular preparation of fluorescent antiglobulin.

The use of successive applications of antisera and fluorescent antiglobulin to identify an unknown organism would facilitate the problem of serological identification of bacteria. The direct technique can be used in a similar manner.

**SUMMARY**

A single fluorescent antiglobulin has been used in conjunction with specific nonfluorescent antiserum (the indirect fluorescent staining procedure) to stain specifically *Brucella suis*, *Pasteurella tularensis*, *Vibrio comma*, and *Pasteurella pestis*. Similar results were obtained in the direct procedure which required a specific fluorescent antiserum for each of the organisms listed. Thus, the indirect procedure accomplished the same results with one instead of many fluorescent preparations. The indirect procedure was employed with both pure and mixed cultures and with small numbers of cells. It was possible to use successive applications of specific antiserum and fluorescent globulin until an identification of the organism was made. Cross reactions were eliminated by employing low titered antisera in both the direct and indirect reactions.

**REFERENCES**


