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Identification of bacteria by specific antibody conjugated with fluorescein isothiocyanate

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INTRODUCTION

When bacteria are allowed to react with homologous immune globulin which has been conjugated with a fluorescein compound, they fluoresce when examined by ultraviolet light (Coons & Kaplan, 1950). This reaction has been used to identify bacteria in smears taken from growing cultures, (Moody, Goldman & Thomason, 1956; Thomason, Moody & Goldman, 1956; Carter, & Leise, 1958; Wolochow, 1959; Winter & Moody, 1959) to detect bacterial antigens in infected tissues, (Hill, Deane & Coons, 1950; Moody & Winter, 1959; Sheldon, 1953), to distinguish individual antigenic components of *Salmonella typhi* (Thomason, Cherry & Moody, 1957), to study the surface antigens of *Haemophilus pertussis* (de Repentigny & Frappier, 1956) and in serological grouping of streptococci (Moody, Ellis & Updyke, 1958).

It should be possible by the fluorescent antibody reaction, to identify a small number of bacteria, at a relatively early stage in their growth, before there is sufficient antigenic material available for performing agglutination tests. The technique would therefore give valuable assistance in rapid identification of slow-growing pathogenic bacteria such as *Pasteurella pestis*, *Past. tularensis* and *Brucella* species.

This paper describes a method of rapid identification of some slow-growing pathogenic bacteria involving:

(i) labelling antisera with fluorescein isothiocyanate instead of the original isocyanate;

(ii) staining impression smears taken from micro-colonies growing on agar.

The influence of various conditions on the intensity of fluorescent staining is also investigated.

MATERIALS AND METHODS

Cultures used

Investigations were carried out on three strains of *Brucella suis*, six strains of *Pasteurella pestis* (three virulent and three avirulent) and one strain of *Past. tularensis*. The *Br. suis* and *Past. pestis* strains were grown on Fildes's agar or on appropriate selective media (Morris, 1956, 1958), and the *Past. tularensis* strain on a blood–glucose–cysteine agar medium similar to that used by Downs, Coriell, Chapman & Klauber (1947).

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Preparation of labelled antisera

Preparation of fluorescein isothiocyanate

Fluorescein isocyanate, the original fluorescent marker used by Coons, Creech, Jones & Berliner (1942), not only reacts readily with hydroxyl and amino groups, but is also readily hydrolysed by water. It seemed advisable to seek a reagent which was less easily hydrolysed and which was also more specific in its action on protein, in order to avoid possible loss of sensitivity in the immune reaction. The isothiocyanates appeared to fulfil both these requirements and accordingly

Table 1

Isothiocyanates

Position of -NCS group	Compound from which prepared	Performance as conjugate
5	5-amino-acridine (*a*)	Weakly fluorescent
5 – *p*	5-*p*-amino-phenylazo-acridine (*a*), (*b*)	Inferior to fluorescein isothiocyanate
5	5-Amino-3-*o*-, hydroxybenzal amino-acridine (*a*)	Caused protein isolubility
3	3-Amino-5-dimethylamino-acridine	
3	3-Amino-5-ethoxyacridine (*a*)	
3	3-Amino-pyrene (*c*)	
?	Aminopyrene trisulphonic acid (*d*)	Inferior to fluorescein isothiocyanate
	p-Amino-phenylazopyrene trisulphonic acid (*e*)	
	β-Naphthylamine 6-8-disulphonic acid	
2	Methyl 2-amino-3-naphthoate	Caused protein insolubility
4′ or 5′	Amino dibromofluorescein	
4′ or 5′	Amino tetrabromofluorescein	

Sulphonylchlorides

Position of substituent group	Compound from which prepared	Performance as conjugate
1	1-Dimethylaminonaphthalene 5-Sulphonic acid	Caused protein insolubility
?	Dimethoxypyrene sulphonic acid (*d*)	

(*a*) A. Albert. *The Acridines*. Edward Arnold and Co. London, 1951.
(*b*) From 5-chloracridine and *p*-nitrophenyl hydrazine and subsequent reduction of the $-NO_2$ group.
(*c*) Vollmann (1957). *Ann.* **531**, 109.
(*d*) Commercial sample.
(*e*) From aminopyrene trisulphonic acid and *p*-nitrosoaniline.

4′- and 5′-isothiocyanato-fluorescein were prepared by the action of thiophosgene on the pair of isomeric amino fluoresceins used by Coons & Kaplan (1950). Both proved quite satisfactory as fluorescent conjugates, a conclusion reached independently by Riggs, Seiwald, Burckhalter, Downs & Metcalf (1958). In order to extend the range of fluorescent markers, a number of other isothiocyanates, pre-

pared by the same general method, and a few sulphonyl chlorides, prepared by the action of phosphorus pentachloride on the corresponding sulphonic acids were examined. The results are summarized briefly in Table 1.

None of the compounds named in the table has been prepared in a state of analytical purity. They have been used in the crude state. As will be seen, none is as good for general use, as fluorescein isothiocyanate. Some, on conjugation with antibody, caused precipitation of the protein, and the remainder were inferior to fluorescein isothiocyanate either in the intensity of their fluorescence, or in the colour contrast with the natural fluorescence of biological material.

Since our method of preparation of fluorescein isothiocyanate differs from that published by Riggs *et al.* (1958), it is described in full.

Amino fluorescein I or II (0·1 g.) prepared by the method of Coons & Kaplan (1950) was dissolved in 5–10 ml. of acetone containing 5% water and added to thiophosgene (0·1 ml.) in 5 ml. acetone. The small amount of water prevents the precipitation of salts of amino fluorescein as a result of the production of HCl in the reaction. The resulting solution was allowed to stand 15 min. at room temperature, evaporated to dryness *in vacuo* and kept in a vacuum desiccator protected from sunlight.

Conjugation with antibody

For most applications it was found desirable to remove the bulk of the immunologically inactive protein (e.g. albumin), from the antiserum. Antibody is usually associated with the γ-globulin fraction and this fraction was isolated by the standard ammonium-sulphate precipitation method, by sodium-sulphate precipitation by the method of Kekwick & Record (1941) or by methanol precipitation, (Dubert, Slizewicz, Rebeyrotte & Machebouef, 1953). It was finally dialysed against saline/phosphate buffer (pH 7·8–8·0), and made up to the original serum volume with the same buffer. To this solution was added an equal volume of a solution of 5 mg./ml. fluorescein isothiocyanate in 3% sodium bicarbonate. The mixture was allowed to stand 24 hr. at 0–5° C. and was then dialysed against saline/phosphate buffer (pH 7·8–8·0) until the dialysate was free from fluorescence. The fluorescein-labelled immune γ-globulin in the dialysis sac was then ready for use.

Technique of the fluorescent antibody reaction applied to growing bacteria

Cultures suitable for testing

The tests were performed on small groups of discrete micro-colonies developing on agar plates after overnight incubation, the diameter of individual micro-colonies ranging from 20 to 100μ.

Method of transfer of bacteria to a glass surface

A $\frac{5}{8}$ in. square glass cover-slip which had been cleaned in nitric acid, rinsed in tap-water, and dried by warming in a flame was placed on the agar surface over the micro-colony or micro-colonies to be tested. The coverslip was gently pressed on

the agar surface, removed with sterile forceps and placed on a glass slide so that the impression of the micro-colonies was uppermost. These impression colonies could be seen at this stage through a binocular microscope at 25 × magnification.

Fixation

The impression was air-dried for about 5 min., and while still on its slide, heat-fixed on a hot plate at 60–65° C. for about ½ min. It was allowed to cool to room temperature and placed in a Petri dish which had been converted into a moist chamber by lining with dampened filter-paper.

Staining

Two drops of conjugated antiserum delivered by 35 drop/ml. capillary pipette were placed on each cover-slip and the serum spread carefully over the whole cover-slip with a wire loop. The preparation was left covered with serum, in the moist chamber, for 5 min. at room temperature.

Washing

The stained cover-slip was removed from the slide with fine forceps, and the conjugated antiserum allowed to drain on to filter-paper. Two or three drops of buffered saline (pH 7·2–7·6) were placed on the cover-slip, which was agitated gently while still held in the forceps. This process, which removed more serum from the cover-slip, was repeated twice, and the cover-slip then replaced on its slide and covered by three or four drops of buffered saline. The impression was left at this stage for 5–10 min.

Mounting

The cover-slip was again removed from the slide with forceps, and the saline drained on to filter-paper so as to leave a thin film of moisture on the cover-slip, which was then placed face upwards on a dry filter-paper. A large loopful of glycerol saline (9 parts glycerol: 1 part 0·85 % saline, buffered at pH 8·2 with phosphate buffer) was placed on the cover-slip which was mounted face downwards on a clean fresh thin slide. In order to facilitate the finding of the correct focus level during microscopy, it was found convenient to draw a grease pencil line on the slide, and place the cover-slip so that the line ran parallel to and just inside one of its edges. The preparation was sealed with paraffin wax.

Optical apparatus

The Cooke, Troughton & Simms apparatus for fluorescence microscopy was used, incorporating a blue filter which restricted illumination to the extreme blue end of the visible spectrum and the near ultraviolet. A yellow sandwich filter, complementary to the blue filter, was inserted in the eye-piece with the thicker glass plate towards the objective.

The microscope was a monocular instrument with substage condenser at full aperture. 16, 4 and 2 mm. oil-immersion objectives and wide field 10 × eye-pieces were used.

RESULTS

Microscopic appearance of bacteria after reaction with homologous labelled antisera

Micro-colonies of *Pasteurella pestis*, *Brucella suis*, and *Past. tularensis*, when treated with homologous conjugated antisera at a dilution of 1/4–1/6, and examined by ultra-violet light, fluoresced brightly, appearing as yellow, granular objects, roughly circular or triangular depending on how far the cover-slip had been dragged over the agar when the impression was taken. The appearances were quite characteristic at the low magnification given by the 16 mm. objective. With the 4 mm. objective, and more so with the 2 mm. oil immersion objective, individual bacteria in the micro-colonies were sharply defined, and with a relatively large organism, such as *Past. pestis*, the fluorescence was concentrated around the periphery of the bacteria and along the septa dividing an apparently bicellular organism, the centre of the cell being left unstained. With 2 mm. objective the phenomenon could also be seen with occasional bacteria in micro-colonies of *Br. suis* and *Past. tularensis*, but owing to the smaller size of the organisms was not so striking as with *Past. pestis* (Pl. 1, figs. 2 and 4). Results were equally convincing whether organisms were grown on selective or non-selective media.

In some preparations isolated single bacteria were seen exhibiting peripheral staining. This indicated that the fluorescent antibody reaction would, theoretically, be capable of detecting a single homologous organism. However, all preparations showed much non-bacterial fluorescent material, derived mainly from the agar, and including many small particles similar in size and shape to bacteria. There was no such confusion between fluorescent micro-colonies and stained non-bacterial matter. The morphological homogeneity of the bacterial cells in an impression microcolony was an important feature distinguishing such micro-colonies from collections of non-bacterial fluorescent objects of similar size and shape.

Specificity of the reaction

The specificity of the reaction was investigated by six different methods.

(i) *Natural fluorescence of an unstained colony*

Bacterial colonies 1 mm. or more in diameter, taken on to a cover-slip by the impression technique, and examined in an unstained, glycerol-mounted preparation by ultra-violet light, were dimly fluorescent, but individual bacteria could not be made out. Colonies below 100μ in diameter could not be seen at all when unstained.

(ii) *Reaction with labelled normal serum*

Micro-colonies of *Past. pestis* stained with conjugated serum taken from unimmunized rabbits (in one case the pre-immunization serum of an animal whose antiserum was used in fluorescent antibody tests on *Past. pestis*), were dimly fluorescent under ultra-violet light, but it was not possible to pick out the sharp

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outlines and peripheral staining of individual bacteria. *Br. suis* and *Past. tularensis* micro-colonies were not even visible by ultra-violet light when stained with normal serum.

(iii) *Reaction with labelled heterologous antisera*

Micro-colonies of different species were treated with labelled heterologous antisera as shown in Table 2.

Two types of result were seen.

(*a*) The treated micro-colonies of *Past. pestis* and *Alkaligenes faecalis* were dimly fluorescent when examined under ultra-violet light, but the definition of individual bacteria was usually poor, and in the few instances where the cell outlines could be made out, a parallel preparation treated with homologous antiserum showed brightly staining and clearly defined bacteria (Pl. 1, figs. 1 and 3).

(*b*) The treated micro-colonies of *Br. suis*, *Past. tularensis* and *Pseudomonas pyocyanea* were quite invisible when examined under ultraviolet light.

Table 2. *Reaction of heterologous labelled sera with micro-colonies of various bacterial species*

Organisms	Labelled antisera	Fluorescence (u.v. light)
Past. pestis	*Br. abortus* *Past. tularensis* *Chromo. violaceum* *Esch. coli*	Dim micro-colonies
Alk. faecalis	*Br. abortus* *Past. pestis*	Poor bacterial definition
Ps. pyocyanea *Br. suis*	*Past. pestis* *Past. tularensis* *Chromo. violaceum.* *Esch. coli* *Past. pestis*	Micro-colonies not visible
Past. tularensis	*Br. abortus* *Chromo. violaceum* *Esch. coli* *Past. pestis*	

(iv) *Detection of specific bacteria in mixed cultures*

Micro-colonies of *Past. pestis* growing in mixed culture with larger colonies of *Alk. faecalis* fluoresced brightly when treated with homologous labelled antiserum. The *Alk. faecalis* colonies stained dimly and showed poor definition. Similarly micro-colonies of *Br. suis* gave a good fluorescent reaction with homologous labelled antiserum in the presence of much larger colonies of staphylococci.

(v) *Inhibition reaction*

Fixed impressions of *Past. pestis* were treated with homologous unlabelled serum for 10 min., buffered saline for 5 min. and specific labelled serum for 5 min., in that order, and then washed in buffered saline and mounted in glycerol saline as before. Fluorescence of these impressions was much less than that shown by

preparations of *Past. pestis* treated with labelled antiserum alone, but was not completely abolished.

(vi) *Reaction of micro-colonies treated with specifically absorbed serum*

Micro-colonies of *Past. pestis* treated with a 1/30 dilution of a labelled homologous antiserum which had been absorbed once with *Past. pestis* before labelling, were less intensely fluorescent than similar micro-colonies treated with unabsorbed labelled antiserum (also 1/30) but brighter than micro-colonies stained with labelled normal or heterologous serum.

It would probably be necessary to absorb a serum three or four times with the homologous organism in order to remove all the antibody, and this should be done with a very concentrated suspension of organisms so that the initial serum dilution could be kept low. Owing to the progressive dilution of the serum during serial absorptions the fluorescent activity of the antiserum might be removed by dilution rather than by specific absorption.

DISCUSSION

The technique described has permitted the specific identification of *Past. pestis*, *Past. tularensis* and *Br. suis* 16–20 hr. after inoculation of agar plates with material containing one or other of these organisms. Identification of these organisms by established methods would require plates to be incubated for 2 or 3 days in order to provide sufficient material for biochemical tests and agglutination reactions.

The impression technique was applied successfully to single micro-colonies of minimum diameter 45–50 μ for *Past. pestis*, 30–35 μ for *Past. tularensis*, and 20–25 μ for *Br. suis*. Smaller minimum diameters were permissible if many colonies of similar morphology were present on the same part of the agar plate.

Micro-colonies of *Past. pestis* growing on cellophane disks overlying agar could be picked up by the impression technique and stained successfully with fluorescent antibody at a slightly earlier growth phase than colonies growing directly on agar. This was probably due to micro-colonies on cellophane being wholly on the surface, whereas those on agar were probably in the early stages, partly beneath the surface (Pearce & Powell, 1951). The micro-colonies on cellophane were nearly always only one cell thick, so that individual bacteria when stained with fluorescent antibody were very well defined.

A drawback to the inoculation of bacteria on to cellophane disks was that the fluid in the inoculum would not soak through the cellophane into the agar sufficiently quickly, and if the cellophane remained too moist, the bacteria would not form discrete colonies but a film of growth which would not be readily visible under low-power microscopy. This was demonstrated with *Br. suis*. On one occasion a cover-slip impression taken 'blind' from a cellophane disk which had been inoculated with *Br. suis* was stained with homologous labelled antiserum; fluorescent bacteria, mainly in ones and twos, were seen when no micro-colonies had been visible on the cellophane before the impression was taken. But it would be impossible to obtain evidence by this method that the fluorescent bacteria had been derived from a *growing* colony.

It is possible, by careful evaporation, to control the amount of moisture on an inoculated cellophane disk so that discrete colonies are formed. The direct staining of cellophane disks with fluorescent antiserum was precluded by the natural fluorescence of cellophane.

Several attempts were made to transfer cells to a cover-slip by a needle or pipette held in a micro-manipulator. This might allow identification to be achieved earlier as only a very small number of cells would be required, but the gain was outweighed by two disadvantages.

(i) A double transfer of bacteria was involved, from agar to needle and from needle to glass surface, with risk of loss of organisms on the needle.

(ii) The micro-colony was broken up and the arrangement of fluorescent bacteria in the stained preparation was not so convincing morphologically as after the impression technique, wherein micro-colony architecture was better preserved.

In practice the fluorescent antibody reaction should be controlled by testing a second set of micro-colonies with labelled normal serum. The identification would then depend upon the micro-colonies treated with homologous antiserum fluorescing much more brightly than those treated with normal serum, and their individual bacteria showing a correspondingly increased sharpness of outline. This would necessitate having sufficient micro-colonies for two tests, which could most probably be ensured by inoculating two plates, or two sectors of one plate, with any material suspected of containing *Pasteurella* or *Brucella* organisms.

It was not practicable to take two successive impressions off the same area of the agar as nearly all the available growth adhered to the first cover-slip. Some impressions were treated first with normal labelled serum, examined under ultraviolet light to ensure that fluorescence of the micro-colonies was negligible, then removed from their carrier slide, and after the glycerol had been washed off, treated with specific labelled antiserum and re-examined.

The micro-colonies fluoresced more brightly after restaining with homologous antiserum than they had done after the initial treatment with normal serum. Also, colonies treated twice with normal labelled serum did not have their fluorescence increased by the second application of serum. But the definition of individual bacteria was relatively poor after the double staining, and 10–15 % of the micro-colonies were lost during removal of the cover-slip.

SUMMARY

1. The reaction of slowly growing pathogenic bacteria with specific fluorescent antibody provided a means for their rapid identification.

2. Fluorescein isothiocyanate was used to label the globulins of antisera as it had a number of advantages over fluorescein isocyanate.

3. Several other isothiocyanates and a few sulphonyl chlorides tested were inferior to fluorescein isothiocyanate as labelling agents. Some of the compounds caused protein precipitation, and others gave a less intense or less distinctive fluorescence than did fluorescein isothiocyanate.

4. The tests were performed by taking impressions of bacterial micro-colonies

on glass cover-slips, drying and fixing the preparations, staining them with labelled antiserum, washing in saline and mounting in glycerol saline. The preparations were then examined microscopically under ultra-violet light.

5. Micro-colonies of *Br. suis*, *Past. tularensis* and *Past. pestis*, when treated with homologous labelled antiserum, fluoresced brightly under ultra-violet light and the individual bacteria were sharply defined and stained at the periphery.

6. Micro-colonies of these and other bacteria stained with normal labelled serum, or heterologous labelled antiserum, showed either very dim fluorescence under ultra-violet light, with poor definition of individual bacteria, or no fluorescence at all.

7. The technique described permitted specific identification of *Br. suis*, *Past. pestis* and *Past. tularensis* within 20 hr. of inoculation of agar plates with material suspected of containing one of these organisms.

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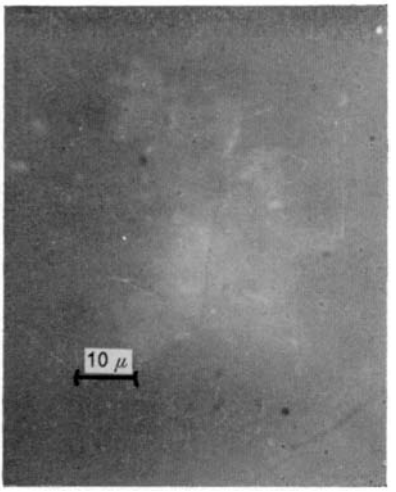
EXPLANATION OF PLATE

Fig. 1. Micro-colony of *Past. pestis* treated with *Br. abortus* labelled antiserum.

Fig. 2. Micro-colony of *Past. pestis* treated with *Past. pestis* labelled antiserum.

Fig. 3. Micro-colony of *Alk. faecalis* treated with *Past. pestis* labelled antiserum.

Fig. 4. Micro-colony of *Br. suis* treated with *Br. abortus* labelled antiserum.



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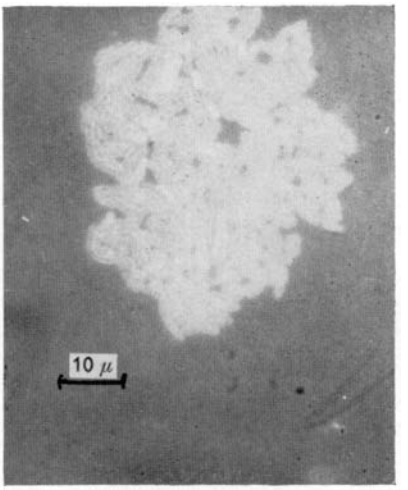

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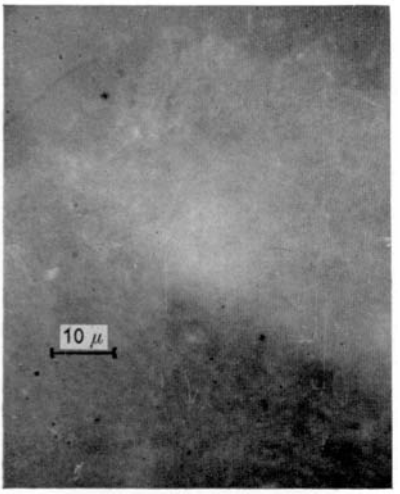

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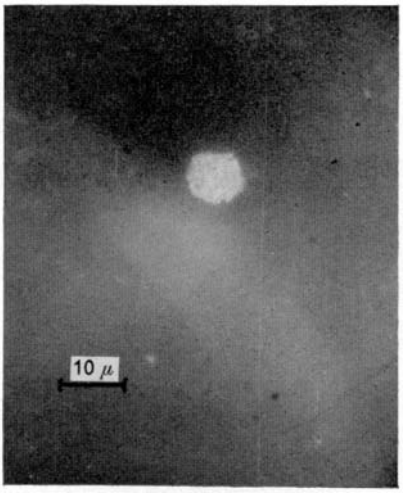

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