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# IDENTIFICATION OF STREPTOCOCCUS AGALACTIAE BY THE FLUORESCENT ANTIBODY

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Fluorescent antibody techniques have been successfully applied to the identification of group A streptococci (*Moody et al.*, 1958, *Wagner* and *Heinrich,* 1962). Rapidity, economy, and ease of performance are cited as advantages of this procedure over the conventional precipitin test. The present investigations were undertaken to test the suitability of the method for the identification of *S. agalactiae,* the main object of bacteriological diagnosis in many mastitis laboratories. This report presents the methods employed and some of the results obtained.

The strains of *S. agalactiae* used for antiserum production were an old reference strain of this laboratory (I) and three fresh isolates (II, III and IV) from milk samples sent to the laboratory for bacteriological mastitis diagnosis. Each of these strains was injected intravenously into one rabbit as a suspension according to the following schedule: 0.2, 0.5, 1.0, 1.5 and 2.0 ml. given every second day, followed by a rest period of 5 days; then 1.5 to 2.0 ml. every second day five times. The rabbits were bled 7 days after the last injection. During the course of further immunisation three of the rabbits died and a later antiserum could be obtained only for strain III. For immunisation, the cells were suspended in saline to give a turbidity of approximately no. 5 Brown standard. Two of the strains (I and II) were injected alive, and two (III and IV) were formalinised (0.3 per cent. final concentration). As the fifth *S. agalactiae* antiserum, a commercial group B specific precipitating antiserum (Behringwerke, D 28/E

1138, Op-Nr. 83) was used. The antibody titres determined by the tube agglutination test for strains I, II and III antisera were from 1:2560 to 1:5120. For strain IV the spontaneous agglutination prevented accurate determination. The titre for strain III antiserum did not change after the additional immunising injections.

Antisera were also prepared in the same manner for one strain each of *S. dysgalactiae* and *S. zooepidemicus* (formalinised antigen).

All these antisera and normal rabbit serum were conjugated with fluorescein isothiocyanate according to the method described in the monograph by *R. C. Nairn* (1962), with the exception that fluorescein isothiocyanate was added in powder form. The conjugates were purified by gel filtration on Sephadex G-25, medium (*Wagner,* 1962).

The bacterial smears for staining were prepared from colonies on blood agar plates. The bacteria were transferred with a loop to a drop of saline on a glass slide and smeared. The smears were allowed to air-dry and fixed gently with heat. One to two drops of the conjugate were applied and the slide allowed to stand for 30 min. at the room temperature in a moist chamber. After quick rinsing, the slides were washed in phosphate-buffered saline (pH 7.4) for 10 min. with one change of the buffer and then allowed to drain. Phosphate-buffered glycerol (90 per cent glycerol, pH 8.4) was used as mounting medium.

The Reichert "Zetopan" microscope with a bright field condensor was used, with an Osram HBO 200 mercury vapour bulb as light source. The filter combination was UG 1/1.5 mm. (primary) and GG 9/1.5 mm. (secondary).

On the basis of the previous experiences reported by other workers (*Moody et al.,* 1958), it was expected that the conjugate of a single *S. agalactiae* antiserum would not stain all *S. agalactiae* strains. Therefore, the four conjugates of the antisera prepared in this laboratory were pooled and the pool used as the principal staining tool for investigations. Twenty-three strains of *S. agalactiae* were stained with both the pool and the four individual conjugates separately. The conjugate of the commercial antiserum was included with the pool in another series of stainings, and still more strains were tested only with the pool. Duplicate slides were always stained with one or more of the control conjugates. All the stainings reported were made with

undiluted conjugates. However, in separate tests it was found that the *S. agalactiae* conjugates could be diluted 1:10 without notable loss in the intensity of fluorescence.

In addition to strains of *S. agalactiae* isolated in routine investigations of milk samples and identified by the CAMP reaction (esculin-negative), other streptococci from the same source were included in the material tested. The smears were examined as a "blind series" to simulate diagnostic specimens. The fluorescent antibody results were compared with the "key" only after the slides had been examined and the results recorded.

Table 1. Staining strains of *S. agalactiae* with individual and pooled *S. agalactiae* antiserum conjugates.

| Conjugate | No. of strains giving different fluerescent antibody reactions | | | | | | | No. of strains tested |
|---|---|---|---|---|---|---|---|---|
| | +++ | ++± | ++ | +± | + | ± | 0 | |
| I | — | 1 | 6 | 4 | 10 | 2 | — | 23 |
| II | 4 | 4 | 3 | 2 | 2 | 3 | 5 | 23 |
| III | — | 1 | 5 | 5 | 7 | 4 | 1 | 23 |
| IV | — | 4 | 8 | 3 | 3 | 3 | 2 | 23 |
| Pool | 2 | 8 | 10 | 3 | — | — | — | 23 |
| Commercial antiserum | — | 1 | 1 | 13 | 8 | 1 | 27 | 51 |
| Pool*) | 13 | 19 | 34 | 13 | 1 | — | — | 80 |

*) Also includes the reactions in the parallel staining with the individual conjugates of the 23 strains given earlier in the table.

Note: Fluorescent antibody reactions are graded with plus and plus-minus symbols as follows:
± = questionable fluorescence; + = definite but faint fluorescence; from +± to +++ = gradual increase in the intensity of fluorescence to very bright greenish yellow staining.

As shown by the results in Table 1, the pool proved to be superior to individual conjugates in its staining properties. Of the individual conjugates, that of strain I showed the broadest spectrum of staining but generally exhibited poor intensity of fluorescence with 2 questionable reactions. Conjugate II, with some intense stainings, entirely failed to stain several strains. This antiserum was weakest in tests for group-specific precipitin. The conjugates of the two strain III antisera were equal in their staining properties. The conjugate of the commercial grouping

serum left approximately half the strains unstained. All the 80 strains of *S. agalactiae* tested by the pool of the conjugates showed fluorescence of moderate to high intensity. In one instance a negative result was at first recorded but afterwards, on closer examination of the slide, a peculiar pattern of staining, very small intensively fluorescing points in the bacterial wall, was revealed. This staining is recorded with + in the table. On some previous occasions, autofluorescing points in the bacterial wall had been encountered, which fact induced the initially erroneous diagnosis in this case. This autofluorescence, however, does not have the same greenish yellow colour as the fluorescein isothiocyanate staining. In the examination of another strain the first trial resulted in a questionable ± reaction but thus raised suspicion of the precence of *S. agalactiae*. In a further test a few days later, the culture having been stored at + 4°C, the strain showed a ++ reaction. One strain recorded as *S. uberis* (CAMP-positive, esculin-positive, non- or α-haemolytic) showed a mixture of fluorescing and nonfluorescing cocci. By further cultural procedures *S. agalactiae* could be isolated from the mixture. Another strain, at first recorded as *S. agalactiae* by CAMP-esculin reactions, was not stained by the fluorescent antibody and was later found to split esculin. Some of the strains of *S. agalactiae* were in mixed cultures with other bacteria than streptococci but could be detected without difficulty with the fluorescent antibody. In no case were *S. agalactiae* strains stained by the controls.

It is a well known fact that *S. agalactiae* strains may have common antigens with group C and G streptococci. Cross-reactions with the representatives of these groups were expected. All the strains (many of them reference strains) of *S. dysgalactiae,* other group C streptococci and group G streptococci, (9, 7, and 4 when tested by the pool) showed varying degrees of fluorescence, in most cases graded +± to ++. However, at the same time they were all also stained by the control conjugate of *S. zooepidemicus* antiserum. The intensity of fluorescence generally corresponded to that caused by the pool. Most of these strains were also stained by the *S. dysgalactiae* conjugate and, surprisingly, even with the normal rabbit serum conjugate. The question of the specificity of these stainings and the problem of why no *S. agalactiae* strains were reciprocally stained by the group C conjugates are under further study. Nevertheless this pattern, staining by both the *S. agalactiae* and control conjugates, eliminated the false positive

diagnosis in these instances. In the preliminary tests the absorption of the *S. agalactiae* conjugate by group C streptococci has been shown to result in abolition of the cross-reactions and in only a slight decrease of the specific staining ability.

Seven strains of *S. uberis*, 5 group E (β-haemolytic), 2 group K and 1 group D strain, were negative to both the *S. agalactiae* and control conjugates. Similar behaviour was shown by 5 strains of bacteria other than streptococci. One strain conventionally recorded as *S. uberis* showed some cocci which were stained by the pool but not by the control in a mixture with nonfluorescing bacteria. The strain was accidentally lost before it could be retested and its identify confirmed.

The results of this series of preliminary experiments seem to justify an increasing interest in the practical application of the fluorescent antibody method in the mastitis laboratory. Further evaluation of the technique on the lines indicated and studies on other aspects of the application are in progress.

## REFERENCES

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## SUMMARY

A fluorescent antibody technic for identification of *Streptococcus agalactiae* is described. Smears from colonies on blood agar plates were tested. A pool of conjugates to four different *Streptococcus agalactiae* antisera stained all the 80 *Streptococcus agalactiae* strains investigated. The pool proved superior to individual conjugates. Also strains of groups C and G were stained by the *Streptococcus agalactiae* conjugates. These, however, could be differentiated from *Streptococcus agalactiae* strains by examination of the controls because the conjugates of antisera to some group C strains stained group C and G strains but not *Streptococcus agalactiae* strains.

## ZUSAMMENFASSUNG

*Identifizierung von Streptococcus agalactiae mit fluoreszierenden Antikörpern.*

Eine Fluoreszenz — Antikörper-Tecknik („fluorescent antibody technic") für die Identifizierung von *Streptococcus agalactiae* wird beschrieben. Ausstriche aus Kolonien auf Blutagar-Platten wurden

untersucht. Eine Mischung „Konjugat" von vier verschiedenen *Streptococcus agalactiae* Antisera färbten sämtliche 80 undersuchten *Streptococcus agalactiae*-Stämme. Die Mischungen erwiesen sich den Einzelkonjugaten überlegen. Stämme der Gruppe C und G wurden auch von *Streptococcus agalactiae*-Konjugaten gefärbt. Diese konnten jedoch von *Streptococcus agalactiae*-Stämmen dadurch unterschieden werden, dass die Kontrollen untersucht wurden. Die Konjugate von Antisera gegen einige Stämme der C-Gruppe färbten nämlich Stämme der Gruppe C und G aber nicht *Streptococcus agalactiae*-Stämme.


## SAMMANDRAG

*Identifierung av Streptococcus agalactiae med fluorescerande antikroppar.*

En fluorescent antibody-teknik för identifiering av *Streptococcus agalactiae* beskrivs. Utstryk från kolonier på blodagar-plattor undersöktes. En blandning konjugat av fyra olika *Streptococcus agalactiae* antisera färgade samtliga 80 undersökta *Streptococcus agalactiae* stammar. Blandningen visade sig vara överlägsen individuella konjugat. Stammar av grupp C och G färgades också av *Streptococcus agalactiae*-konjugaten. De kunde dock skiljas från *Streptococcus agalactiae-stammarna* genom att undersöka kontrollerna. Konjugaten av antisera emot några grupp C stammar färgade nämligen grupp C och G stammar men inte *Streptococcus agalactiae*-stammar.


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