METHOD FOR DEMONSTRATING CAPSULES OF ENTEROBACTERIACEAE

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The wet film India ink method (Duguid, 1951) has satisfactorily demonstrated capsules and slime substance in capsulated species of the genera: *Diplococcus, Klebsiella, Escherichia, Shigella,* and *Bacillus* (Duguid, 1951; Edwards and Fife, 1952; Ewing *et al.*, 1951). This method fails to show capsules on strains of *Escherichia coli* which have so-called "B" and "L" envelope antigens (Kauffmann, 1954) in young broth cultures grown at 37 C (Orskov, 1956).

The quellung test of Neufeld for demonstrating capsules on diplococci and Klebsiella (Etinger-Tulczynska, 1933; Klieneberger-Nobel, 1948; Duguid, 1951) does not work too well for capsules on certain serological types of *E. coli.*

When cultures of *E. coli* with the B antigen are added to undiluted homologous antisera, the size of the bacterial cells increases and the light refraction becomes more intense. However, a capsule cannot be discerned because the outline of the protoplasm is not visible. The presence of a capsule is deduced only by comparing the size of bacterial cells in homologous antiserum with those in normal or in heterologous antiserum.

The purpose of this paper is to describe a simple staining technique for capsules of *E. coli* having B and L surface antigens.

MATERIALS AND METHODS

Cultures. Strains of *E. coli* with B surface antigens (group O: 111, 55, 26, 86, 114, 119, 125, 126, 127, and 128) were obtained from Dr. F. Kauffmann and Dr. Frits Orskov in Copenhagen and Dr. J. Taylor in London. Duplicate strains isolated in Djakarta were also used. A strain of *E. coli* having L surface antigen (strain F 103 (025: 11L:H6)), was obtained from Dr. Frits Orskov, and similar strains (233/55 (025:.L:H16) and 133/55 (025:.L:H6)) were isolated in Djakarta. Controls consisted of a strain of *E. coli* Bi 449 (09:K26:H..) obtained from Dr. Frits Orskov and a Klebsiella, both having A surface antigens, and *Shigella boydii* type 1 with B surface antigen.

Culture method. Two transfers on meat extract agar plates were made to obtain characteristic smooth opaque colonies; 18-hr cultures were then inoculated into glucose broth previously prepared as follows. Two-tenths ml of sterile 20 per cent glucose was added to 10 ml of broth of the following composition: distilled water, 1100 ml; NaCl, 3 g; Na_2HPO_4 (anhydride), 1 g; tryptose (Difco), 10 g; meat extract (Difco), 10 g; pH 7.6. Cultures were incubated at 37 C for 4 to 8 hr.

Preparation of antisera. The method of Kauffmann (1954) was employed. Saline suspensions of 18-hr cultures on glucose agar plates were used for making antisera for organisms having B or L surface antigens. Cultures (6 to 8 hr old and treated with formalin) in glucose broth were used for preparation of antisera for organisms with A surface antigens.

Stain solution. Alkaline methylene blue (Colour Index no. 922) Loeffler's stain (Gradwohl, 1948) was used. The solution was kept no longer than 2 months in a clean, dark colored, glass stoppered bottle and for each day's use about 0.5 ml was placed in a small tube.

Procedure. A loopful (about 0.01 ml) of undiluted homologous antiserum is placed on a chemically clean glass slide, and an inoculating needle point of broth culture is added but not mixed. A chemically clean cover glass is carefully placed on the slide so that edge *a-d* (figure 1) came in contact with the mixture. After several seconds the cover glass is lowered slowly to allow the fluid to spread evenly underneath and to avoid air bubbles in the mixture. No pressure should be applied to the cover glass even if a large drop of serum is used. Allow the preparation to stand at room temperature for ½ hr (Djakarta room temperature is 28 to 31 C and the humidity is never less than 50 per cent). On standing, the preparation becomes viscous and Brownian movement and motility of flagellated organisms can still be observed.

After ½ hr, remove the cover glass by care-

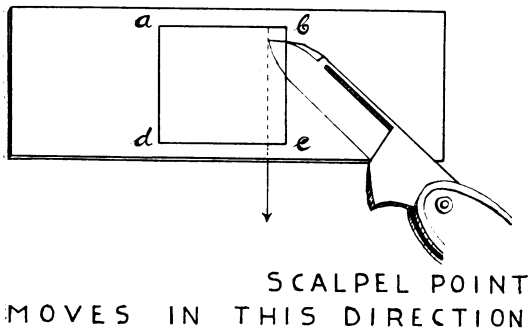

Figure 1. Technique for removal of cover glass.

fully pushing the tip of a sharp scalpel between the cover glass and slide. If the cover glass is firmly attached, the scalpel should be moved slowly to prevent breaking the cover glass. Add 1 drop (±0.01 ml) Loeffler's stain to the preparation on the slide so the fluid flows to *a-d.* Replace the cover glass in the manner previously described, and examine the preparation under the oil immersion objective.

RESULTS

Observation of capsules. Immediately after adding stain there is a gradual adsorption of dye by cells. The cytoplasmic membrane at the poles becomes blue, then bluish-purple, and soon the entire cytoplasm becomes bluish. At this time the capsule is not visible with either direct or oblique illumination. The size of all stained cells is the same as in unstained preparations with homologous antiserum.

After 3 to 5 min the cytoplasm of organisms with large capsules, such as Klebsiella, *E. coli* with A surface antigens, and *Shigella boydii* type 1, and bacteria with smaller capsules, such as *E. coli* O group: 111, 55, 86, 26, 114, 119, 125, 126, 127, and 128, assume a deep violet color and appear intensely refractile. Cells appear large and glistening in direct illumination but no capsule is visible. With oblique illumination a delicate refractile capsule with a dark outline lying close to the cytoplasmic membrane can be observed after 5 to 10 min. The capsular material itself may not stain but when it does, this usually requires at least 20 min. The capsular substance appears either light blue or light violet and the capsular outline stains light blue to light purple. Capsules can be demonstrated only when the organisms are in homologous antiserum.

There is no difference between organisms isolated in Indonesia and standard strains from Europe when the organisms are used in the demonstration of capsules in the serum-broth stain preparation.

The capsular outline around the dark violet cytoplasm remains visible for a long time if the surrounding fluid does not dry out. Contraction of the cytoplasm does not occur after 1½ hr observation.

When an inadequate quantity of stain is added or when stain is added too soon to the serum-broth mixture, the cytoplasm sometimes fails to stain. In such preparations, a second drop of methylene blue may be added 20 min after the first drop. Usually the protoplasm and the outline of the capsule become visible in a short period of time.

Organisms with thin capsules, such as old isolations of *E. coli* O group 86, may require the addition of 1 drop of distilled water to the preparation, 20 min to ½ hr after the methylene blue has been added. The fluids are mixed by tilting the slide and the same or a clean cover glass is again placed on the preparation in the same manner as previously described. The staining time of the capsular material of *E. coli* O group 86 is usually prolonged to 1 or 1½ hr.

Observation of slime substance. In 8- to 10-hr broth cultures, the standard capsular swelling reactions with Klebsiella, *E. coli* strain possessing A surface antigens, and a weak capsulated *E. coli* O 86 show slime precipitates as refractile threads radiating from the cell surface and sometimes as bundles of fine hairs lateral or polar from the cells. In 18- to 24-hr cultures, the slime precipitates are abundant and appear like a reticulum of branched fibers in which the cells are discerned with difficulty.

Using this staining method on cultures 18 to 24 hr old, the slime stains light blue. The slime structure changes with time becoming granulated, similar to the granular slime structure reported by Duguid (1951) in dry film preparations with eosin-serum as staining background.

In the present investigation, 43 strains of *E. coli* with B surface antigens and 1 strain of Shigella show capsule and slime formation. It is necessary to use young cultures to demonstrate capsules and older cultures (18 to 24 hr) to demonstrate slime. The slime can be demonstrated only when the organisms are in homologous antiserum.

Observation concerning O sera. When homologous antisera prepared with 2½ hr steamed broth cultures were used in the above method, capsule demonstration was identical to that of preparations in homologous antisera prepared with living organisms. *S. boydii* type 1 also showed capsules when homologous antiserum made with heated vaccine was used.

Observation on capsules of E. coli strains with L surface antigens. Using homologous antisera prepared with living cells, the three strains of *E. coli* having L surface antigen readily showed capsules. This is an unusual finding since these cells do not appear to enlarge distinctly in unstained preparations with homologous antiserum. The capsular outline appears within 5 min and the capsular substance itself usually stains after 20 min. In time the color grows paler, but this was never observed in organisms having B antigens.

When a drop of water is added to preparations 20 min after staining, the capsular outline disappears in strain F 103 (which has flat colonies on agar) and the slime precipitates around the cells. But when water is added to similar preparations of strains 233/55 and 133/55 (which have raised colonies on agar), the capsules enlarge greatly and assume the morphology of a slime envelope. Capsules can be demonstrated best on organisms from broth cultures 2 to 4 hr old, and rarely after 5 hr. No capsules can be demonstrated with homologous O antisera prepared with 2½-hr steamed broth cultures.

DISCUSSION

When a drop of Loeffler's methylene blue stain is added to a serum-broth mixture and the preparation is observed after ½ to 1 hr, most of the cells remain colorless and capsules are not seen. The size of the bacteria remains unchanged, but with more concentrated stain, the cells shrink. Previous spreading and aging of the serum-broth mixture under a cover glass allows the dye to be adsorbed more rapidly and consequently a change occurs in the protoplasmic structure of cells.

A weak alkaline Loeffler's methylene blue is preferred because it causes no shrinkage of the bacterial protoplasm, whereas other basic dyes such as methyl violet and Nile blue, although having strong affinity for bacterial protoplasm, are too toxic and cause a decrease in cell size and plasmolysis.

Aging of the serum-broth films at room temperature (28 to 31 C) with a humidity of 50 to 70 per cent gives good results. This is not due to the temperature but to the humidity of the environment. Preparations standing for ½ hr at 35 C in a dry incubator failed to show capsules, whereas preparations standing in an incubator of 35 C with a humidity of 60 to 70 per cent readily showed capsules.

While the dye molecules are diffusing through the slime layer and are binding with the cytoplasmic material, the capsular outline is invisible because of the similar refractive index of capsular substance and environment. The outline becomes visible when the adsorption of the dye is completed and the difference of refractive index of the jelly slime layer and surrounding fluid becomes greater.

The addition of distilled water to stained preparations of small capsulated organisms is necessary since changing the refractive index of the system is the only way to make the capsular outline visible. Water can be added several times to preparations of *E. coli* with B antigens since it causes only weak lysis of the capsule. The capsular outline becomes fibrilar but, in time, again becomes smooth. Preparations wrapped in paper and stored overnight in the refrigerator dry out, but will again show capsules when a drop of water is added.

Concentrated meat-extract broth stimulates slime production more than ordinary broth containing 0.6 per cent meat extract. The slime of the two strains with A antigens appears in the former medium after 5 hr incubation whereas in the latter broth only after 8 hr. Prolonged steaming (1 to 1½ hr) of the medium prior to autoclaving also stimulates slime production.

Many media have been recommended for stimulating slime production by bacteria (Duguid, 1951; Edwards and Ewing, 1957; Orskov, 1956), but too much slime will obscure the capsule. This observation has been made on broth cultures of *E. coli* with B antigen when incubated longer than 10 hr. The slime becomes abundant and capsules are not seen. This is probably due to the masking effect of the slime which surrounds the capsule and prevents a difference in light refraction. In stained preparations after ½ hr the refractility of the slime masses is pronounced as a result of greater refractive index due to coagulating processes. Organisms in coagulated slime

appear larger because the material acts to magnify the size, and the capsular outline is not seen. Edwards and Fife (1952) reported failure of capsular reactions in capsulated Klebsiella in the presence of slime precipitates. Therefore, they recommended use of a pinpoint amount of suspension for capsular reactions for diagnostic purposes. Masking effect caused by precipitated slime has also been noticed by Perch (1950) in a mucoid culture of *Salmonella paratyphi* B. Observing freshly prepared capsular preparations of suspensions from agar culture in broth mixed with homologous antiserum, she found that the capsular swelling was not typical, but after aging the preparations she found large distinct capsules.

The present study confirms other observations that capsules are best demonstrated in young cultures, such as in studies on mucoid streptococci by Morison (1940). Kauffmann (1949) recommended the use of young broth cultures for microscopic capsular reactions for the slimy Klebsiellae. Ewing *et al.* (1951) have used 4-hr broth cultures for quellung reactions of *S. boydii* 1 and 2. Perch (1950) has used 8-hr broth cultures grown at 20 C for capsule demonstration of 2 mucoid cultures of *S. paratyphi* B. In one very mucoid culture she found that the demonstration failed if the broth culture was older than 8 hr. According to Perch the phenomenon was due to autolysis of the capsule. In the present investigation using 43 *E. coli* having B antigens, 3 with L antigens, 1 with A antigen, 1 Klebsiella, and 1 Shigella strain with B antigen, it has been found that each organism has its own period of capsulation; that is, an optimal time for forming a well defined capsular layer without too much production of loose slime. Weak capsulated strains have the shortest period of capsulation and for the detection of their capsules the examination should begin with 2-hr broth cultures.

The sensitivity of the staining method has been demonstrated by positive capsular reactions of *E. coli* with B surface antigens in their homologous O sera. Using only the quellung reaction it has been impossible to prove the presence of real capsular antibodies in the O sera of this group of *E. coli* (Orskov, 1956). The presence of capsular antibodies in O sera, however, was assumed by Kauffmann and Dupont (1950) and Kauffmann (1954) because of the partial agglutinations found in homologous O sera with smooth living antigens. Four criteria for defining *E. coli* with B antigens have been presented by Orskov (1956). Another very important criterion, which discloses a true capsular nature is presented by the use of this new staining method, i. e., that the antigenic property of the B antigens is not, or only weakly destroyed after heating at 100 C for 2½ hr.

Recent studies have shown that this method is useful in the identification of capsular antigens and in the determination of mutual relationships between colon bacteria with A, B, and L antigens. Determination of the capsule titers of sera have been done with all sera used in this investigation: both A capsule sera had titers of 1:8, whereas the general capsule titer of the B sera was 1:3 to 1:4. The 3 L sera had titers: undiluted, 1:5, and 1:2, respectively, for F 103, 233/55, and 133/55.

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SUMMARY

A sensitive method for demonstrating capsules of *Escherichia coli* with A, B, or L surface antigen is described.

The incubation of the organisms in homologous serum-broth mixture under a cover glass in a humid environment is essential prior to the addition of stain.

Organisms must be young in order to demonstrate the capsules free from slime precipitates.

Capsules of *E. coli* with B surface antigens can be demonstrated in homologous antiserum prepared both with living and heat-killed organisms.

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