Simple Method for Distinguishing Gonococcal Colony Types

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Gonococcal colony types can be distinguished by a new procedure that makes use of a dissecting microscope with a concave mirror and a fluorescent lamp. Critical adjustment of the mirror angle results in illumination similar to that obtained in the dark-field microscope. When the concave mirror is set at a certain angle, colonies of the lenticular types 1 and 2 refract the light coming through them in such a way that an edge of the microscope stage is focused in each colony. By contrast, colonies of types 3 and 4, which are relatively flat, fail to refract incident light. Although distinguishable from each other by differences in color, type 3 and 4 colonies do not display the focusing effect typical for type 1 and 2 colonies and appear uniformly illuminated. This new technique permits the rapid identification and isolation of even a single type 1 or 2 colony in a field of type 3 or 4 colonies, making it possible to obtain and maintain competent colonies (type 1 or 2) for the genetic transformation assay for *Neisseria gonorrhoeae* strain identification as well as for other purposes.

Neisseria gonorrhoeae has been shown to undergo a relatively rapid variation of unknown origin that results in the appearance of several different colony types when cultures are plated on suitable transparent media and observed with a dissecting microscope under special conditions of illumination (4, 8). Strains of *N. gonorrhoeae* freshly isolated from infected individuals give rise predominantly to colony types 1 and 2, whereas cells producing only colony types 3 and 4 are most frequently found in cultures that have been maintained in the laboratory by repeated subculture (5, 9, 13). It is possible, however, to maintain colony types 1 and 2 by using special media (9). This colony type variation is of considerable significance since only cells from colony types 1 and 2 are pathogenic; cells derived from colony types 3 and 4 are unable to initiate infection in human volunteers (7, 8). Unlike cells from colony types 3 and 4, cells from colony types 1 and 2 are piliated (5, 10, 14) and are also highly competent for genetic transformation (3, 12).

It is usually not possible to distinguish *N. gonorrhoeae* colony types by examining colonies with the naked eye. Colony types have been differentiated with low-power dissecting microscopes with either diffuse and angled transmitted light (1, 2, 7, 8) or with a double system of angled substage lighting (4). During recent studies in our laboratory leading to the development of a genetic transformation assay for identification of *N. gonorrhoeae* strains (3), it was necessary to be able to distinguish colonies of com-

petent cells (types 1 and 2) from colonies of noncompetent cells (types 3 and 4). By using a stereoscopic dissecting microscope, we discovered a simple and highly effective method for observation and differentiation of the *N. gonorrhoeae* colony types. The present communication describes this new procedure and illustrates the appearance of the various colony types of *N. gonorrhoeae* by this method.

MATERIALS AND METHODS

Organisms and media. Most of the *N. gonorrhoeae* strains examined in the present study were derived from clinical specimens obtained from the diagnostic laboratory of St. Joseph Mercy Hospital, Ann Arbor, Mich. A few strains were obtained from the American Type Culture Collection, Rockville, Md. The photographs (Fig. 2 and 4-7) show the various colony types of strain 488, a uracil auxotroph that is used as a tester strain in the genetic transformation assay for *N. gonorrhoeae* strain identification (3). All cultures were stored in 67% glycerol at -45°C as previously described (6). The transparent medium used to grow and observe the various colony types in the present study was medium M1 (3). Other media suitable for colony type observation include the GCB, GCBB, and GC8-2DS media of Kellogg et al. (8), and medium M9 (3). Cultures were incubated at 35 to 36°C in a candle jar, usually for 16 to 18 h. Photographs were taken through a Bausch and Lomb StereoZoom 7 microscope at magnifications of $\times 20$ to $\times 50$ with a Bausch and Lomb photomicrographic camera and Polaroid type 107 film.

Identification of colony types. The various *N. gonorrhoeae* colony types were determined initially by the procedure of Jephcott and Reyn (4).

RESULTS AND DISCUSSION

Basic method for observing colony types. The method to be described was originally developed with a Bausch and Lomb StereoZoom 7 microscope. A small modification must be made when other microscopes are employed. It is important that a concave mirror be used for best results. However, it is possible to use a flat mirror. The basic method will be described for use with the Bausch and Lomb microscope with a concave mirror. The modification required for use with other microscopes and a procedure making it possible to use a flat mirror will then be given.

The plate to be observed is illuminated with a fluorescent lamp adjusted so that light reaches the substage mirror both from above and from below the microscope stage (Fig. 1A). Focus the microscope on colonies on a plate and remove the plate from the stage. Do not refocus the microscope during subsequent steps of the procedure. Rotate the concave mirror so that it is

horizontal and facing up. The field now observed through the unrefocused microscope should be dark. While looking through the microscope, slowly rotate the mirror toward the light source. As rotation is continued, a bright field of illumination will appear. This is followed by a dark field that is then followed by a second bright field. Upon observing the second bright field, stop rotating the mirror. Carefully reverse the direction of rotation, turning back into the dark field, until the approximate center of this dark field is reached. At this point, the mirror angle is adjusted for critical illumination. The dark field results from the blockage of light by the solid edge of the microscope stage, indicated by the arrow in Fig. 1A. At this angle, all the light reaching the concave surface of the mirror is reflected from it to the stage at various angles, none being directed vertically into the microscope. Although the empty field appears dark at critical illumination, objects that reflect or refract the angled light will appear brightly illuminated. Any scratches on the glass stage, for example, will appear as bright lines.

Figure 2A shows the appearance of a mixture of type 2 and 3 colonies of *N. gonorrhoeae* 488 on a plate that was placed on the microscope stage after critical illumination was achieved as described above. The lenticular (double-convex-shaped) type 2 colonies (7, 11) act as small lenses and focus the edge of the microscope stage (arrow in Fig. 1A) in each colony so that it appears as a dark horizontal bar in the center of a brightly illuminated colony. Minute adjustment of the mirror angle will serve to center this dark bar in each colony if the original adjustment did not result in perfect centering. At this point, it is possible to check the lamp angle for optimum positioning. The bright areas above and below the dark bar in each type 2 colony should be equally right. If this is not the case, the angle of the lamp should be changed to obtain equal colony illumination. It will be noted that adjustment of the lamp angle is not nearly as critical as adjustment of the mirror angle. By contrast with type 2 colonies, type 3 colonies will be relatively dimly but fairly uniformly illuminated and will not have central horizontal dark bars (Fig. 2A).

To verify further that the horizontal dark bar observed in each type 2 colony (Fig. 2A) was indeed due to focusing of the stage edge (arrow in Fig. 1A) in the colony, the colonies shown in Fig. 2A were viewed with a vertical metal rod placed in the path of the light directed toward the mirror (Fig. 1B). The results of this change in illumination are shown in Fig. 2B, in which each type 2 colony now has focused in it the vertical rod as well as the horizontal microscope

FIG. 1. Illumination used with the Bausch and Lomb StereoZoom 7 microscope. (A) Position of fluorescent lamp for critical illumination. The arrow indicates the opaque microscope stage edge discussed in the text. (B) Illumination with a vertical metal rod (arrow) in the light path.

FIG. 2. Appearance of a mixture of type 2 and 3 colonies of strain 488 with critical illumination. (A) In this typical view, all type 2 colonies have dark central bars in contrast to the type 3 colonies, which are uniformly illuminated. (B) The same colony mixture illuminated with a vertical bar in the light path. (C) The same colony mixture illuminated with the vertical bar extending down only to the edge of the microscope stage. (D) The same colony mixture photographed with random illumination.

stage edge. When the vertical rod was raised so that it extended down to the edge of the microscope stage, and not below, each type 2 colony only focused the vertical rod in its top half (Fig. 2C). When no attempt was made to obtain the critical mirror angle and essentially random illumination was used, the type 2 and 3 colonies appeared as shown in Fig. 2D.

Critical illumination with microscopes other than the Bausch and Lomb microscope. The critical illumination just described was obtained readily with Bausch and Lomb StereoZoom 7 or Series B microscopes, so it came as a surprise to find that critical illumina-

tion could not be achieved with an American Optical Cycloptic microscope equipped with a concave mirror. Further investigation of this situation revealed that differences in the dimensions of the stage edge (arrow in Fig. 3A) and relative placement of the mirror mounting in the American Optical microscope resulted in inability to obtain complete dark-field illumination with this unit. This situation could be compensated for, however, by a small increase in size of the effective light-blocking edge of the microscope stage. This was accomplished by placing an opaque object, such as the ruler shown in Fig. 3B, at the edge of the glass stage

FIG. 3. Illumination used with the American Optical Cycloptic microscope. (A) The arrow indicates the opaque microscope stage edge discussed in the text. (B) Position of fluorescent lamp for critical illumination with a microscope stage edge extender (ruler) as discussed in the text.

nearest the lamp. In the absence of this stage edge extender, and at the mirror angle corresponding to critical illumination, it could be seen that the microscope field was not entirely dark, bright areas being visible at the top and bottom of a central dark area. With the stage edge extender in place (ruler in Fig. 3B), the complete field was dark at critical illumination.

With the ruler in place and the mirror now adjusted for critical illumination, a mixture of type 2 and 3 colonies appeared exactly as they do in Fig. 2A. Once the best position for the ruler was determined, the ruler could be replaced by a piece of opaque tape, such as black plastic electrical tape. This strip of tape is not visible through the microscope and may be left in place permanently, since it will not interfere with other applications of the microscope.

One of the dissecting microscopes encountered during this study has a U-shaped metal frame supporting a rectangular sheet of glass with no opaque edge. Placement of an opaque

strip, such as a ruler or a piece of opaque tape, at the edge of the stage nearest the lamp served to make it possible to achieve critical illumination. In this case, as well as the one previously described, the opaque tape can remain in place permanently without interfering with other uses of this microscope.

Modification required for use of a flat mirror. When a flat mirror was used in place of a concave mirror, it was found to be impossible to obtain critical illumination, since only a narrow dark band was visible even with the Bausch and Lomb microscopes. Upon making the field as dark as possible by adjustment of the mirror angle, a mixture of type 2 and 3 colonies appeared as shown in Fig. 4A. Although the two types of colonies are different in several ways, both of them have horizontal dark bars through their centers (Fig. 4A). It was possible to darken the entire field by extending the stage edge of the Bausch and Lomb microscope with a ruler in a manner similar to that illustrated in Fig. 3B. With this modification, the more extreme extension of the opaque microscope edge resulted in relatively little light being able to reach the flat mirror with the lamp in the position shown in Fig. 1A. By simply rotating the lamp 90° so that more light could reach the mirror from both above and below the extended stage edge, it was possible to remedy this situation. The picture in Fig. 4B shows the same group of colonies seen in Fig. 2A and 4A, with the stage edge extended and the lamp rotated 90° as indicated above. Under these conditions of illumination, type 2 colonies have characteristic dark bars through their centers, whereas type 3 colonies do not (Fig. 4B).

Some microscopes are routinely supplied with only a flat mirror, the opposite side usually being a flat diffuse surface. It is possible to modify such microscopes by taping a concave mirror from an old unused, conventional high-magnification microscope to the flat mirror or the flat diffuse surface. Although the flat mirror can be used successfully to distinguish gonococcal colony types, as illustrated above, the concave mirror is distinctly superior and warrants either the modification just described or purchase of a concave mirror when none is available.

Observation of type 3 and 4 colonies. Type 3 and 4 colonies of *N. gonorrhoeae* are well known to be interconvertible in many strains (4, 7, 8). Figure 5A shows an artificial mixture of type 3 and 4 colonies of *N. gonorrhoeae* 488. Critical illumination of the kind described above is, therefore, also useful in enabling differentiation of colony types 3 and 4. Although both colony types are essentially the same size, type 3 colonies are distinctly whiter

than type 4 colonies, the latter being relatively more transparent. It may be somewhat difficult to be certain about the identification of some type 4 colonies when viewed alone, since colonies of this type from a particular strain may be somewhat whiter than type 4 colonies from another strain. When the colony type 3 to 4 inter-conversion takes place, however, it is always possible to recognize the type 3 colonies as the relatively whiter ones. When *N. gonorrhoeae* is plated on the medium M1 used in these studies, colony types 3 and 4 are somewhat larger than colony types 1 and 2 (see Fig. 2A). Figure 5B

shows the same group of type 3 and 4 colonies as in Fig. 5A, photographed by the technique described above that employs a flat rather than a concave mirror. Although it is possible to distinguish colonies of type 3 from colonies of type 4 in Fig. 5B, the use of the concave mirror (Fig. 5A) is clearly superior.

Colonies of type 4 of certain *N. gonorrhoeae* strains have been shown to revert only rarely to colonies of type 3, whereas type 3 colonies of these strains revert very rapidly to type 4. In such cases, it is almost impossible to maintain a fairly uniform population of type 3 colonies.

FIG. 4. Appearance of the same mixture of type 2 and 3 colonies of Fig. 2, seen through the Bausch and Lomb StereoZoom microscope with a flat mirror. (A) Critical illumination without a microscope stage edge extender. (B) Critical illumination with a microscope stage edge extender.

FIG. 5. Critical illumination of a mixture of type 3 and 4 colonies of strain 488. (A) Illumination with a concave mirror. (B) Illumination with a flat mirror and microscope stage extender as described in the text.

In other strains, however, it is possible to maintain populations of either type 3 or 4 colonies because of the relatively low rate of interconversion of colony types in either direction.

Differentiation of colony types 1 and 2. Colony types 1 and 2 are both characterized in the present system of illumination by a horizontal dark bar in the center of each colony (Fig. 6). Colonies of type 2 (Fig. 6B), which are brighter than colonies of type 1, have sharp-edged horizontal bars, whereas the horizontal bars of colonies of type 1 (Fig. 6A) have edges that are considerably less sharply defined. Colonies of type 2 are significantly more translucent than colonies of type 1, the type 1 colonies being more transparent. Furthermore, the horizontal bars in colonies of type 1 tend to be darker than those of type 2. It is easily possible to distinguish colonies of type 1 from colonies of type 2 when these factors are kept in mind.

Appearance of areas of confluent colonial growth. Previous studies of gonococcal colony types with other methods of illumination have emphasized that colony type differentiation is best made for colonies that are isolated and not confluent (2, 4, 8). Figures 6A and 6B show the appearance of confluent growth areas as well as isolated colonies of colony types 1 and 2, as viewed with the critical illumination of the present procedure. As the colonies become more crowded, the horizontal bars in colonies of types 1 and 2 become thicker and are occasionally curved. Confluent growth areas have the same shade of darkness as the bars in the individual colonies. The typical horizontal bars are still evident in colonies that are very close

to areas of confluent growth. Isolated colonies of types 3 and 4 are uniform in appearance (Fig. 5A) under the present system of illumination, and the color of the colonies does not change when growth becomes confluent.

Relationship of colony types 1 and 2 to colony types 3 and 4. As a result of careful studies of colony type interconversion of strain 488, we have been able to confirm the observation of Jephcott and Reyn (4) that colony type 2 mutates chiefly to colony type 3 and colony type 1 mutates chiefly to colony type 4. Although the reverse mutations to colony types 1 and 2 occur less frequently, we have found that type 3 colonies give rise to type 2 colonies and type 4 colonies give rise to type 1 colonies. Colonies of type 1 do occur rarely in a population of type 2 colonies, and these type 1 colonies have been isolated and shown to breed true. When starting from a population of type 2 colonies, perhaps the simplest way to obtain a population of type 1 colonies is to select and streak out one of the type 3 colonies that appear spontaneously at a fairly frequent rate. Subculture of type 3 colonies will eventually reveal type 4 colonies that also arise by spontaneous mutation. A type 4 colony is then subcultured and will in time give rise to colonies showing horizontal bars when viewed with critical illumination, such colonies invariably being of type 1.

It should be emphasized that although colonies of types 1 and 2 mutate to colonies of types 4 and 3, respectively, the ability to show the reverse mutation is strain specific, some strains being able to revert readily whereas other strains

FIG. 6. Confluent growth of colony types 1 and 2 of strain 488, viewed with critical illumination. (A) Colony type 1. (B) Colony type 2.

FIG. 7. Critical illumination of a mixture of colony types 1, 2, 3, and 4 of strain 488.

revert very poorly or not at all during extensive subculture. Strains able to revert colony type 3 to colony type 2 are also able to revert colony type 4 to colony type 1.

Figure 7 shows the appearance of an artificial mixture of colony types 1, 2, 3, and 4 as viewed with critical illumination. Although all the photographs shown are of colonies of strain 488, many other clinical isolates of *N. gonorrhoeae* have also been studied by the present technique. Except for minor differences, colony types of other strains were similar in appearance to the colony types of strain 488.

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