

Variation in Colonial Morphology of *Neisseria gonorrhoeae* After Growth on Media Containing Antimicrobial Agents

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Stable colonial types 1, 2, 3, and 4 were prepared from eight strains of *Neisseria gonorrhoeae*. Four of the strains, termed laboratory strains, had been transferred over 100 times; three strains, termed clinical strains, were transferred only three to five times after isolation from patients, and one stabilized clinical strain was transferred purposefully 30 times after isolation from a patient. Colonial types of the three categories were grown on four media containing the following agents at the level used in diagnostic media: (i) vancomycin, colistin, and nystatin; (ii) these antibiotics plus trimethoprim lactate; (iii) trimethoprim lactate alone; and (iv) a control with no antimicrobial agents. When grown on media containing the antimicrobial agents, colonial types 1, 2, and 3 of all strains showed specific and consistent changes that precluded accurate identification of the types. In general, the colonies were smaller, more dense to transmission of light, and more granular than colonies grown on control medium. More colonies showed these type changes in the clinical strains and on media containing trimethoprim lactate. Colonies of type 4 showed little or no change. The changes in colonial morphology of types 1, 2, and 3 were pronounced enough to make colony typing difficult if the antimicrobial agents, particularly trimethoprim lactate, were present in media.

Correlation between virulence and colony type of *Neisseria gonorrhoeae* was first reported by Kellogg et al. (1, 2) in experimental infections of man. An important extension of this correlation would be determination of colony types isolated from cases of gonorrhea. However, cultivation of *N. gonorrhoeae* from patients is usually done on media such as Thayer-Martin (8) or modified Thayer-Martin (4, 11) which contain inhibitory agents to prevent growth of bacteria that may be present in specimens along with *N. gonorrhoeae*. The effect of these inhibitory agents on the colonial morphology of types of *N. gonorrhoeae* and on the accuracy of colony typing procedures has not been extensively documented.

Colony typing is a subjective procedure, and colonies of *N. gonorrhoeae* freshly cultured from patients are particularly difficult to type. Therefore, for our study we used strains whose colony types were known and stabilized. The strains were grown on media containing antimicrobial agents representative of the kinds and amounts used for clinical isolation procedures. The resulting colonies were compared with

controls grown on media without the inhibitors, and the morphological changes were recorded.

MATERIALS AND METHODS

Cultures and isolation of colony types. Certain characteristics of the gonococcus are affected by the number of transfers on laboratory media after initial isolation (9, 10). Therefore, to evaluate changes in morphology that might result from transfer, as well as from exposure to inhibitory agents, eight strains of *N. gonorrhoeae* were used which varied in number of transfers in the laboratory from as few as three to more than 100. These strains were originally isolated on Thayer-Martin or modified Thayer-Martin media (4, 8, 11). Initial identification was made by gram stain and oxidase reaction with final confirmation by sugar fermentation reactions and by fluorescent antibody tests. All cultures were incubated in candle jars at 36 C for 24 h.

Laboratory strains F-62, Ngc98, Ngc113, and Ngc117, from the Center for Disease Control (CDC), had been transferred more than 100 times. Strain F-62 has been used as a control by many investigators (1, 6, 7).

The clinical strains EE, VJ, and WOR were isolated from genital exudates of patients seen at the City of Houston Social Hygiene Clinic. After initial

culture on Thayer-Martin medium, they were maintained on control medium. These strains were transferred three times, the minimal number necessary to separate the colony types.

The stabilized clinical strain WP was handled like the clinical strains, except that the separated colony types were transferred purposefully 30 times. After this number of transfers, the colony types were stable with few changes from types 1 or 2 to types 3 or 4. When our initial experiments were completed, the three clinical strains also were transferred 30 times to produce stabilized clinical strains for comparison with strain WP.

Colony types 1, 2, 3, and 4 (2) were isolated from each strain. For identification of the types, each strain was grown on control medium that did not contain hemoglobin (2, 5, 11). A typical representative of each of the four colony types was identified for each strain and transferred to fresh control medium. A minimum of three transfers after initial isolation was required to get pure clones of each colony type. The colony types of each strain were frozen in a solution of Trypticase soy broth (BBL)-glycerol (4:1) and stored at -60°C until needed.

Media. Hemoglobin was deleted from all media because a transparent medium was required for typing colonies (2, 3, 5). Control medium contained GC base (BBL) plus 1% defined supplement (IsovitaleX, BBL). VCN medium contained the same kinds and amounts of antimicrobial agents as used in Thayer-Martin medium (8), i.e., vancomycin (3.0 $\mu\text{g}/\text{ml}$), colistin (7.5 $\mu\text{g}/\text{ml}$), and nystatin (12.5 U/ml). VCN-T medium had these same three inhibitors plus trimethoprim lactate (5.0 $\mu\text{g}/\text{ml}$). This combination of antimicrobial agents is found in modified Thayer-Martin medium (4, 11), which now is used widely for

isolation of *N. gonorrhoeae* because trimethoprim lactate inhibits *Proteus* sp. The T medium contained only trimethoprim lactate (5.0 $\mu\text{g}/\text{ml}$), and, although this medium is not used for cultivation of *N. gonorrhoeae*, it was used in our experiments to determine the effects of trimethoprim lactate alone. All media had a pH of $7.3 \pm .05$.

Identification of colony types and photography. Colony types were examined and identified by the technique of Kellogg et al. (2), which required transparent medium to permit passage of light. The colonies were observed by using a stereoscopic microscope (American Optical Stereo Star zoom microscope with auxiliary lens attachment number 574) with the light from the illuminator (American Optical Starlite) angled upward through the petri plate from a frosted glass reflector set at an angle of 120° opposite the illuminator.

Photographs were made on Panatomic X film with a Zeiss photoscope with a $2.5\times$ objective. The condenser was removed, and the aperture diaphragm was adjusted for proper illumination for photography. Final prints had a magnification of $40\times$.

RESULTS

Our experiments are summarized graphically in Fig. 1, and typical changes in colonial morphology are shown photographically in Fig. 2, 3, and 4. When colonial types 1, 2, and 3 were grown on media containing the antimicrobial agents, colonial morphology changed for strains prepared from all three categories of *N. gonorrhoeae* (Fig. 1). Most marked alterations in the greatest number of colonies occurred on

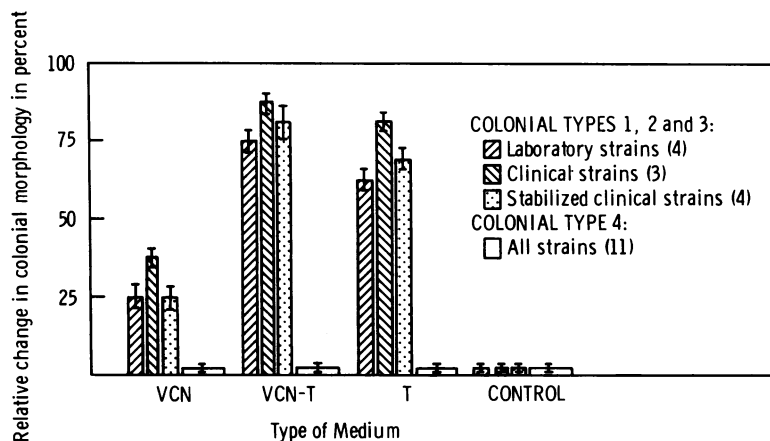


FIG. 1. Relative number of colonies showing changes in colonial morphology of *Neisseria gonorrhoeae*, types 1, 2, 3, and 4, prepared from laboratory, clinical, and stabilized clinical strains, after growth on media containing various antimicrobial agents. The stabilized clinical strains include the three clinical strains transferred purposefully 30 times. The width of each bar indicates the number of strains examined in each group; vertical lines indicate the variation in change within each group. VCN indicates vancomycin, colistin, and nystatin; VCN-T, VCN plus trimethoprim lactate; and T, trimethoprim lactate alone. The control medium contained no antimicrobial agents. Cultures were incubated at 36°C in a candle jar for 24 h before examination. These data were obtained by examining 50 colonies per plate on each of two replicate plates. Changes from normal size (25–50% reduction) and composition such as granularity and density occurred together, so degree of change was recorded on the ordinate as percentage of colonies affected by the inhibitors.

media containing trimethoprim lactate, but moderate changes also occurred on VCN media. The greatest amount of alteration occurred in clinical strains, but repeated transfer of these bacteria in the laboratory lessened the changes (Fig. 1). Laboratory strains transferred more than 100 times showed the least amount of change. The morphology of colonial type 4 from all strains was not changed by growth on any of the media (Fig. 1).

The changes in colonial morphology were similar whether the types were prepared from laboratory, clinical, or stabilized clinical strains. Media containing trimethoprim lactate produced the greatest number of alterations (Fig. 1). Since VCN-T medium is similar to modified Thayer-Martin medium, now widely used for isolation of *N. gonorrhoeae* from patients, we selected this medium to document photographically changes in colonial morphology. Appearance of colonies growing on VCN-T medium was compared with growth on control medium that contained no antimicrobial agents.

Type 1 colonies were small, about 0.5 mm in diameter, raised, with an entire edge, and with a smooth surface (Fig. 2). Different strains varied somewhat in appearance, although type 1 colonies could be recognized easily from all strains. Such variation can be seen between colonies of F-62 and WP (Fig. 2a and c). After growth on VCN-T medium, type 1 colonies became more granular, smaller, and more dense to passage of light (Fig. 2b and d). In some cases, the increase in granularity was so pronounced that the colony resembled a type 3 (Fig. 2d, arrow).

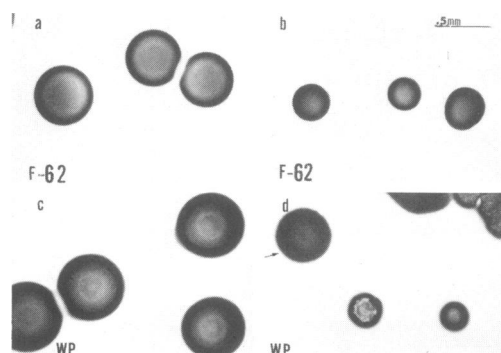


FIG. 2. Colonial type 1 of a laboratory strain, F-62, and a clinical strain, WP, of *N. gonorrhoeae* growing on control medium (a and c) and VCN-T medium (b and d). The arrow in b indicates a change in morphology in which a type 1 colony appears like a type 3. Cultures were incubated at 36 C in a candle jar for 24 h.

Type 2 colonies, like type 1, were small in size, but they had raised centers and flattened edges. This shape caused type 2 colonies to transmit light like a lens and gave a characteristic black band around the edge of each colony (Fig. 3). The colonies were rough rather than granular. In contrast to type 1 colonies, type 2 colonies did not become more granular after growth on media containing antimicrobial agents but appeared heaped and lumpy, rough, more dense, and smaller in size (Fig. 3b and d). These changes were more marked, and appeared in more colonies, for clinical strain VJ than for laboratory strain F-62 (compare Fig. 3b with d).

Types 3 and 4 are different from types 1 and 2 in that they lack pili and are avirulent (1, 7). Both type 3 and type 4 colonies, shown together in Fig. 4, were larger in size (about 1 mm in diameter) than were types 1 and 2. Type 3

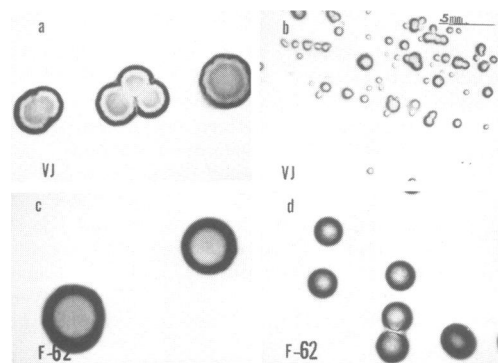


FIG. 3. Colonial type 2 of a laboratory strain, F-62, and a clinical strain, VJ, of *N. gonorrhoeae* growing on control medium (a and c) and VCN-T medium (b and d). Cultures were incubated at 36 C in a candle jar for 24 h.

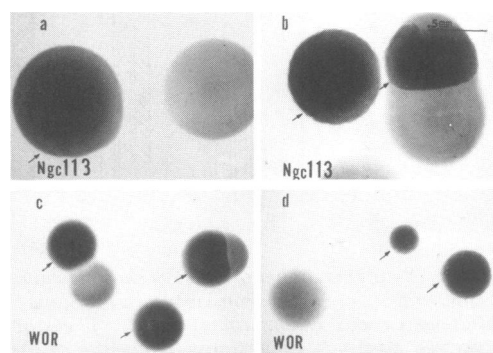


FIG. 4. Colonial types 3 (indicated by arrows) and 4 of a laboratory strain, Ngc113, and a clinical strain, WOR, of *N. gonorrhoeae* growing on control medium (a and c) and VCN-T medium (b and d). Cultures were incubated at 36 C in a candle jar for 24 h.

TABLE 1. Changes in appearance of colonial types of *Neisseria gonorrhoeae* after growth on media containing antimicrobial agents^a

Colony type	Change ^b
1	+ Granularity + Density - Size
2	+ Density + Heaping + Roughness - Size
3	+ Granularity + Density + Irregular edges - Size
4	± Change

^a Agents added to media were (i) vancomycin, colistin, and nystatin; (ii) these antibiotics plus trimethoprim lactate; and (iii) trimethoprim lactate alone. See text for concentrations added to the media. Changes were noted after growth at 36 C in a candle jar for 24 h.

^b Increase in characteristic, +; decrease, -; little or no change, ±.

colonies were granular and light brown in color. When grown in the presence of antimicrobial agents, the colonies became smaller, with more irregular edges, denser, and more granular (Fig. 4, arrows). These changes were so pronounced that a small type 3 colony could be mistaken for a type 1 (middle colony, indicated by arrow, Fig. 4d).

Colonial type 4, rarely isolated from patients, was a flat, colorless, transparent colony with few distinguishing features. The morphology of these colonies showed little or no change when grown on media containing antimicrobial agents (Fig. 4).

DISCUSSION

The low levels of vancomycin, colistin, and nystatin present in Thayer-Martin medium altered the colonial morphology of gonococcal types, and the addition of trimethoprim lactate effected marked changes (Fig. 1). Although typing of colonies is dependent upon visual recognition and is therefore subjective, the changes were pronounced enough that even experienced investigators would be unable to type colonies accurately. Cultures most recently isolated from patients showed the greatest number of colonies with changes in colonial morphology, a fact that should be considered by

investigators interested in determining the colonial types of *N. gonorrhoeae* in such specimens.

The changes in appearance of the various types are summarized in Table 1. The degree of change varied slightly from strain to strain and was affected by the number of transfers after isolation of cultures. The number of colonies showing change decreased the greater number of times a culture was transferred but never disappeared entirely for types 1, 2, and 3.

Our investigations extend those of Kovalchik and Kraus (3) and include not only cultures recently isolated from patients but also cultures transferred many times in the laboratory. Both studies indicate that investigations of colonial types of *N. gonorrhoeae* must be done on media to which no antimicrobial agents are added. In addition, investigators should use for such studies cultures whose colonial type is known so that variations brought about by cultural conditions can be detected.

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