Use of Ultraviolet Light and Fluorescent Dyes in Bacterial Counting. (17843)

LAURENCE W. GREENE, JR., AND H. CLOSE HESSELTINE.

From the Department of Obstetrics and Gynecology, University of Chicago Clinics, and the Chicago Lying-in Hospital, Chicago, Ill.

It is the purpose of this preliminary paper to introduce a new, improved method of counting bacteria. The bacteria are stained with fluorescent dyes and counted in the Petroff-Hausser chamber and illuminated by a reflected ultraviolet light. Ultraviolet light and fluorescent dyes have been used previously in detecting tubercle bacilli(1,2) and protozoan parasites(3). Attention was focused upon the further use of ultraviolet light and fluorescent dyes in studying bacteria by observations noted in connection with the detection of cancer cells of vaginal smears as performed by Friedman(4) in this institution. The clarity of the mixed bacterial flora suggested the applicability of this technique to the bacterial counting chamber.

Apparatus. The General Electric AH4 mercury vapor lamp was mounted in a lamphousing. This lamp was operated from a special transformer. A UG4 Schott filter (Fish-Schurman Corporation, New York City) 6 m in thickness was placed in front of the lamphousing lens system. The smooth surface of the substage glass microscope mirror should be replaced by an aluminum faced mirror as the latter has a higher reflectivity for ultraviolet light. A protective Eastman Kodak Wratten 2A filter is inserted into a standard monocular microscope. The Wratten filter is used for the purpose of filtering out any stray ultraviolet rays while simultaneously allowing the visible fluorescent light to pass freely through the eyepiece. (Fig. 1) A Petroff-Hausser counting chamber with Neubauer ruling No. 4101-A was used in this work. It was difficult to pick up the Neubauer rulings on the counting chamber from this source of light. Therefore, a special Petroff-Hausser counting chamber was obtained with the Neubauer rulings etched with deeper lines.

Method. A bacterial suspension to be counted is drawn up into the Thoma dilution pipette for red blood cells to the 0.5 mark. This is diluted to the 101 mark above the bulb with a 1/1000 dilution of the desired fluorescent dye, thus making a 1:200 dilution in the bulb itself. Should the growth of organisms be scant, a dilution of 1:20 is made by means of the No. 3392 Thoma White Cell pipette. The pipette is shaken in the usual manner to break up clumps and to make an even distribution of the bacteria throughout the suspension. The suspension is left for 5 minutes to allow the bacteria to take up the stain. Four to 6 drops of the suspension are allowed to escape through the

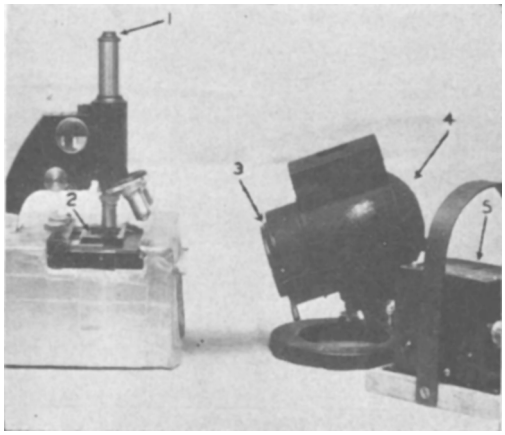

FIG. 1.

1. Microscope ocular with Wratten 2A filter inserted.
2. Bacterial counter.
3. UG4 Schott filter.
4. Lamphousing with General Electric AH4 mercury vapor lamp.
5. Transformer.

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2. Levinson, L. and White, R. L., *New England J. Med.*, 1947, v237, 186.
3. Bock, E. and Oesterlin, M., *Zentralbl. f Bacht.* (abt. 1), 1939, v143, 306.
4. Friedman, H. P., Jr., *Am. J. Obstet. and Gynec.*, 1950, v59, 852.

TABLE I

Response of Selected Organisms to Fluorescent Dyes when Exposed to Ultra-Violet Light

	Sarcina lutea	Strep. viridence	Strep. pyogenes	Staph albus	Staph aureus	Proteus X-19	C. diphtheriae mitis	A. aerogenes	E. coli	Shigella shiga	Sal. paratyphi B	Sal. paraty phi A
Acid Fuchsin												
Acridine Orange	3*		3	2	1	2	2	1	1			2
Acridine Yellow	1	3	2		3	2	3	3	3	1	1	3
Acriflavin	2	2	2		3			3	3	2	2	2
Alizarin Red	1	1	1							1	1	
Auramine O	1	1	1	1	1	1	1	1	1	1	1	1
Berberine Sulfate	1	1	1	1	1	1	1	1	1	1	1	1
Congo Red												
Eosin Y				3	3		2	3	3			3
Fluorescein												
Methylene Blue												
Neutral Red												
Phosphine 3R	1	1	1	3	3	3	3	3	3	1	1	2
Primulin Yellow	1	1	1	2	2	1	1	1	1	1	1	2
Proflavin	3	3	3	3	3	3	3	3	3	3	3	3
Rhodamine B	1	1	1	1	1	1	1	1	1	1	1	1
Thioflavin S	1	1	1	3	3	3	3	3	3	1	1	3

*1 Excellent 2 Good 3 Fair Blank - Unsatisfactory

tip of the pipette and discarded after which the counting chamber is loaded in the routine manner so that the ruled area is covered without overflow into the moat. The room should be moderately darkened for best results. Four to 6 minutes must be allowed for the mercury lamp unit to reach a uniform ultraviolet light production. The count is then made. When viewed through the ocular of the microscope, the bacteria are seen to fluoresce brightly and distinctly against a black background.

Results. 17 different fluorescent dyes were investigated in varying pH of 5.0 through 7.0. (Table I). Fluorescent dyes are photosensitive and should be protected from visible light when not in use. The following microscopic organisms (The first 12 organisms were obtained through the courtesy of the Department of Bacteriology, University of Chicago) have been used and have been found to be very satisfactorily counted by this technic:

1. *Salmonella paratyphi* A; 2. *Salmonella paratyphi* B; 3. *Shigella shiga;* 4. *Escherichia coli;* 5. *Aerobacter aerogenes;* 6. *Staphylococcus aureus;* 7. *Staphylococcus albus;* 8. *Sarcina lutea;* 9. Proteus X-19; 10. *Corynebactor diphtheria* (mitis); 11. *Streptococcus pyrogenes;* 12. *Streptococcus viridans*; 13. *Trichomonas vaginalis;* 14. *Candida albicans.*

There were 3 dyes in this series which were superior with the organisms studied; namely: Auramine-O, Rhodamine-B, and Berberine Sulfate. A fourth dye, Primulin Yellow was found to vary from good to excellent depending upon the bacterial strain. (Table I). Berberine sulfate was preferred in this study because of the relationship of color and background contrast. Numerous bacterial counts were made using both visible and fluorescent light sources on identical cultures. The comparative value is very simply illustrated by the following example. Four samples from a thoroughly suspended culture of *E. coli* were taken in immediate succession. The fluorescent dye was used as the diluent. The counts were made first with visible light and then the identical squares were counted with the ultraviolet light. The average count using visible light was 192,400,000 bacteria per milliliter. The average count under ultraviolet light was 307,000,000 bacteria per milliliter. The single counts made under visible light ranged from 160,000,000 bacteria per milliliter to 240,000,000 bacteria per milliliter, whereas the same fields counted making use of the ultraviolet light ran from 300,000,000 to 320,000,000 bacteria per milliliter. Thus more bacteria could be counted in equal dilutions with the ultraviolet light and fluorescent dyes than with ordinary light. The counts made using this new technique averaged less than one minute for actual counting time as compared with several minutes when the standard visible light was employed.

Summary. 1. The use of ultraviolet light and fluorescent dyes as an adjunct to the Petroff-Hausser bacterial counting chamber technique is reported.

2. This method has made bacterial counts more accurate because of clarity, ease, and rapidity.

3. Berberine Sulfate was the dye of preference although Auramine-O and Rhodamine-B were gauged equally as good.

4. Twelve strains of bacteria, one protozoa, and one fungi were used in testing this procedure.

5. All 14 organisms gave excellent fluorescent reaction to 3 dyes and 11 strains gave excellent results to a fourth dye.

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Response of the Pig to APF, B_{12} and B_{12b}. (17844)

J. E. BURNSIDE, T. J. CUNHA, H. M. EDWARDS, G. B. MEADOWS, G. A. LAMAR, A. M. PEARSON, AND R. S. GLASSCOCK. (Introduced by Paul B. Pearson.)

From the Department of Animal Husbandry, Florida Agricultural Experiment Station, Gainesville, Fla.

Using a corn-peanut meal ration Cunha *et al.*(1) showed that an animal protein factor supplement (Lederle) and crude concentrates (charcoal and fuller's earth) of B_{12} differed in their effect on the pig. The objective of this experiment was to determine what the effect of a relatively pure concentrate of B_{12} and B_{12b} is when given orally or when injected and to determine whether or not the effect is different from that of APF.

Materials and methods. The control ration used consisted of corn, peanut meal, minerals, and the known vitamins which the pig has been shown to need as previously described by Cunha *et al.*(1) Four 8-week-old pigs, equalized with respect to sex, condition, weight, thriftiness and previous dietary history were allotted to each lot. The feeding period was for 40 days. The pigs were housed and fed on concrete floors which were washed once daily.

Results and comments. The data obtained in this trial (Table I) show that vitamin B_{12} (a mixture of B_{12} and B_{12b}), fed either orally or injected did not significantly affect the rate of growth when added to the corn-peanut meal basal ration for a feeding period of 40 days. This confirms earlier data by Cunha *et al.*(1) when concentrates (charcoal and fuller's earth) of B_{12} were fed orally. The pigs injected with B_{12} in lot 2 did not gain as well as the pigs on the basal ration. The pigs in lot 3, fed APF and injected with B_{12}, did not gain as rapidly as the pigs fed APF only in lot 5. Thus, in the 2 lots of pigs, where B_{12} was injected, it seemed to decrease the rate of gain about 0.1 lb. daily.

A diarrhea was observed during the first 2 weeks of the trial in the pigs on the basal ration and periodically throughout the trial in the pigs in lots 2 and 4 where vitamin B_{12} was used. No diarrhea was observed in lots 3 and 5 where APF was added to the ration. Thus, these and other observations obtained with this basal ration at this Station, show that APF contains some factor or factors which prevents a diarrhea (semi-liquid yellowish feces) in pigs. The APF supplement (lot No. 8108-109 prepared by drying the mycelium from deep aerobic cultures of *Streptomyces aureofaciens* together with some filter aid) was found to contain 3 mg of aureomycin per gram by microbiological assay at Lederle Laboratories. Jukes *et al.*(2) and Cunha *et al.*(3) showed that either aureomycin or APF prevented diarrhea with

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