Quantitative Infrared Photoanalysis of Selected Bacteria

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Received for publication 3 December 1973

A technique to measure transmitted infrared radiation from minute biological systems is described. Infrared color film was exposed by radiation transmitted through bacterial colonies. The resultant photographic image was unique for each species of bacteria examined and spectral analysis of the image provided differential light emission patterns which could be quantitated. A formula for developing numerical comparisons among bacterial colonies was provided. The results of this numerical procedure gave quantitative relationships for the total infrared data from each microbial colony and made possible the differential identification of ten species of medically significant bacteria.

Of all differences among bacterial species, none are more generally relied on for identification than cellular morphology and metabolism (2). These characteristics are demonstrated through variations of colony color, texture, density, geometry, rate of growth, gas evolution, heat liberation, and chemical composition. The collective effect of these features produces unique patterns of absorption or reflectance in response to interaction with the electromagnetic spectrum (3, 4). A number of studies (8, 9) have shown that infrared spectrophotometry can be used to distinguish between various genera of bacteria. However, these procedures are time consuming and demand specialized equipment not readily available in nonresearch and clinical laboratories. This paper describes results of an infrared analysis of bacterial colonies which may have future application in the clinical laboratory.

MATERIALS AND METHODS

Organisms and media. Bacteria from the Brigham Young University culture collection were maintained on agar slants consisting of 12% beef heart infusion (Difco), 1.2% peptone, 1.2% gelatin, 0.6% dextrose, 0.6% casein, 0.5% disodium phosphate, 0.4% sodium citrate, and 0.9% agar. The transparent medium used to obtain transmitted infrared (TIR) data consisted of 1.6% nutrient agar, 0.25% yeast extract (Difco), and 0.25% glucose. A 20-ml amount of medium was placed into disposable petri dishes (100 by 15 mm). The medium was inoculated with approximately 50 bacterial cells of the desired strain, and the cells were distributed evenly over the agar surface. All cultures were incubated at 37°C.

Infrared photomicrography. Figure 1 is a schematic diagram of the instrumentation used for infrared recording of bacterial colonies. A Leitz Photolab II photomicroscope was fitted with a 35-mm camera back and further modified as follows. (i) Light emanating from the tungsten source was enclosed in a light-tight corridor to the microscope stage (Fig. 1, item E). (ii) The stage was modified such that light filters could be imposed between the light and the object (Fig. 1, item D). A Leitz brightfield microscope condenser was used with both aperture diaphragms wide open. One eyepiece was fitted with a micrometer for centering the object and measuring bacterial colonies under examination.

A Kodak (Rochester) number 87 infrared filter was positioned in the light path between the source and the object (Fig. 1, item D). This filter allows transmission of radiation from 720 nm to beyond 1,500 nm. Power to the light source was controlled by using a filament transformer equipped with an ammeter and voltmeter. Ektachrome infrared film (Kodak) having a single emulsion series number was stored at 4°C in moisture-tight containers prior to use.

Infrared recording procedures. IR film to be standardized was allowed to come to room temperature (22°C) before placing it in the camera back. Power source was equilibrated and a petri dish containing culture media was placed on the microscope stage. The microscope was focused on the agar surface through the camera window. The power was adjusted to 1 A and the film was exposed by using an A.S.A. of 100 and a shutter speed of 1/4 s. Subsequent exposures were made at 0.25 A power increments up to 8 A. The film was then developed and used to demonstrate film characteristics (indicated in Fig. 3, 4, and 5). Each subsequent role of film was compared to this standard to insure proper control of exposure and development procedures.

Each roll of film must be standardized to correct for variation in the media and photographic procedures. This standardization is accomplished by re-
moving the number 87 filter and focusing the optics on the agar surface. The filter is replaced and two frames of film are exposed, one at 3.5 W of power and the other at 8 W. When these control frames have been exposed the filter is again removed and the bacterial colony to be analyzed is brought into focus under the microscope. The filter is replaced and final focusing is done through the camera by using the colony edge for proper planar adjustment. Because colony morphological detail is not required, it is unnecessary to make focal length adjustment for changes in colony height. The power is adjusted to a value between 6.5 and 8.0 W to obtain proper exposure of the film, and the colony is photographed. Development of the film is done immediately after exposure.

Exposed IR film was developed by using Kodak E-4 processing (6). Fresh Kodak developer solutions were used to develop only four rolls of film and then were discarded. Constant film-chemical agitation was maintained during the development procedures. Recommended film development times and water bath temperatures were held constant (7).

A 10-mm wide strip was cut from the center of the photograph of the desired colony. This picture was then attached to a 35-mm photographic film leader 10 cm long and 10 mm wide. The sample and leader were then threaded into the scanning assembly of a Beckman DBG spectrophotometer (Fig. 2). The spectrophotometer was fitted with a 25-cm chart recorder set to linear response. With the light wavelength set at 580 nm and by using a beam width of 1.5 mm, the sample was passed in front of the light beam at 2.5 cm/min. The density-geometry pattern of the photographed colony was recorded on the chart paper.

For infrared analysis, the scanner as shown in Fig. 2, items B and D, was replaced with two identical open sided film cuvettes. With these cuvettes, there is no surface interposed between the film and the light source. The reference cuvette should contain Kodak number 16 density filter. The photograph of the bacterial colony is placed in the sample cuvette, and, with the light wavelength set at 780 nm, the wavelength scan is activated and completed at a wavelength of 380 nm.

This procedure is, in principle, a comparison between two infrared (IR) photographs of identical agar surfaces, one of which includes a bacterial colony. The difference in appearance, as seen be the IR film, provides a means for characterization of the colony. This difference is measured by using a scanning spectrophotometer to reveal subtle changes in the layers of dye on the film.

RESULTS

Kodak Ektachrome IR film contains three layers of visible and infrared-sensitive film dyes. Exposure results in red coloration as a function of the incident infrared intensity.
Therefore, this film provides both color and density change as a function of the infrared properties of the photographed bacterial colonies. Figure 3 shows analysis of a plain transparent agar plate photographed at two infrared intensities. The data from such samples are in response to the transmitted IR which has only been attenuated by the transparent agar. These data serve as standards for peak positions and intensities which can then be used for analytical comparisons. Two characteristics result from analysis of these recordings: (i) peak area, which is designated as the static infrared unit (SIRU) and is determined by dividing the area under the peak (Fig. 3, peaks 1 or 2) by 100, and (ii) wavelength of peak transmission. In photoanalysis the film negative records the amount of specifically reduced dyes as a function of both IR wavelength and the IR intensity. The infrared intensity recorded on the film is determined by transmission of a corresponding wavelength of visible light through the photograph in the spectrophotometer.

Figure 4 represents change in SIRU of peaks 1 and 2 as a function of infrared power incident upon the film surface. Peak 2 is representative of infrared above 700 nm, whereas peak 1 represents shorter wavelengths. The SIRU of peak 1 is inversely related to the wavelength of infrared radiation, whereas the SIRU of peak 2 is directly related and becomes minimal when infrared transmission is very low or nonexistent. Concomitant with changes in SIRU values is a shift in the wavelength of maximum transmission, i.e., as a function of IR intensity. Figure 5 shows the positions of peaks 1 and 2 (refer to Fig. 3) as a function of the power of infrared transmission.

Each experiment can be controlled by the following procedure. The curves in Fig. 5 represent SIRU values as a function of 0.5 W increases in power up to 25 W. The tangent of the angle between the linear portion of the curve and the abscissa is designated as gamma. This gamma value then may serve as a means for standardization of results obtained for each roll of film from the same emulsion series. The SIRU values obtained from the first two control frames taken on each roll of film, (those at 3.5 and 8 W) are plotted and the resultant gamma value serves as a correction for reading the SIRU values of test organisms photographed on the same film roll at any IR intensity. The gamma value is characteristic for individual
emulsions and experimental conditions and should be obtained for each roll of film that is used. Should a recording be made at a variety of infrared intensities on a particular roll of film, any standard SIRU value can be found from this data. As each roll is its own reference, the significance of the control standard is to give an indication of how far from an optimal reproduction any particular film roll is.

Data obtained by this technique of transmitted IR recording provides two types of information about the bacterial colonies. (i) a spectrophotometric scan of the photographed bacterial colony gives information which is highly characteristic for many species. (ii) Analysis of the photographs of bacterial colonies gives an indication of the alterations in the IR radiation that is absorbed by the colonies as compared to the unattenuated IR radiation of an appropriate agar standard. Figures 6 (Escherichia coli) and 7 (Neisseria catarrhalis) show the results of a spectrophotometric analysis of IR photographs of the indicated bacterial colonies. These data were obtained after 18 h of bacterial growth.

The “A” portion of these figures is the result of a spectrophotometric scan of the colony done at 580 nm. Variations in the recorded pattern (Fig. 6 and 7A) result from differences in colony geometric configuration and are obtained by passing the film through the spectrophotometer operating at the indicated wavelength.

The transmission recordings (Fig. 6 and 7B) are obtained by keeping the film stationary in the spectrophotometer and scanning the photograph over a range of wavelengths of light. In these figures it should be kept in mind that the peak areas represent the greatest cell density in the colonies. The recorded IR pattern in these figures is mediated by colony composition as it interacts with radiation from the illuminating source.

Figure 8 shows the changes in infrared transmissions of bacterial colonies as a function of incubation time. The change in infrared transmission with age indicates change in geometry, and composition which collectively affect the colony’s ability to absorb, transmit, and reflect IR radiation. However, such transmission change was not obvious for all of the organisms tested. Much of the final characteristic of the transmission patterns was not developed until late in the colony growth process. It was also
apparent that reproducibility and ease of species identification increased with the age of the colony.

The transmission and geometric patterns provide three characteristics which are used to determine quantitative values: (i) geometric conformation (Fig. 6 and 7A); (ii) the SIRU (Fig. 6 and 7B); and (iii) peak positions (Fig. 6 and 7B). Using the values from these three characteristics it is possible to construct a table showing quantitative relationships for each species of organism examined (Table 1).

In Table 1, two characteristics of the infrared transmission recording are combined into one value called beta. The position of peaks 1 and 2 as well as the SIRU for each peak is determined from the recorded transmission curve (Fig. 6 and 7B). The values thus obtained are used in the following formula to obtain beta. $\beta = (x - s)/(t - y)$ where $\beta$ equals beta value; $x$ equals (SIRU of peak 2 from the standard) + (wavelength of maximum transmission of peak 2 from the standard); $s$ equals (SIRU of peak 2 from the test organism) + (wavelength of maximum transmission of peak 2 from the test organism); $t$ equals (SIRU of peak 1 from the test organism) + (wavelength of maximum transmission of peak 1 from the test organism); and $y$ equals (SIRU of peak 1 from the standard) + (wavelength of maximum transmission of peak 1 from the standard).

The structure of the equation was derived in its present form as a result of attempts to determine the ratio most useful in indicating maximum differences between sample values and suggests no other relationships.

Table 1 shows the characteristic alpha and beta values for each test organism. Bacteria as closely related as *Streptococcus pyogenes* and *Streptococcus salivarius* show similar alpha values but show distinguishingly characteristic beta values, whereas *Shigella sonnei* and *E. coli*, two closely related enteric organisms normally distinguished and identified only by biochemical tests, give easily differentiating alpha values.

![Fig. 7. IR photoanalysis of Neisseria catarrhalis colony. (A) Geometric-density recording of the colony surface. (B) Photoanalysis indicating film response to transmitted IR radiation from bacterial colony. Data were obtained by using a Beckman 10-inch linear-log recorder operated at 10 cm/min.](image)

![Fig. 8. Photoanalysis indicating film response to transmitted IR radiation from E. coli colonies at 5, 12, and 25 h of growth.](image)
Table 1. Quantitative data resulting from photoanalysis of selected bacteria

| Organism          | Geometric | Infrared |
|-------------------|-----------|----------|
|                   | Base *    | Alpha *  | Peak 2 | Peak 1 † | Beta * | SD ‡ |
| N. catarrhalis    | 10        | 1/9.3    | 196/740| 1271/595| 2.88   | 0.17  |
| S. typhi          | 32        | 3/21.1   | 792/735| 2210/600| 1.29   | 0.46  |
| S. typhimurium    | 12        | 3/54.5   | 1411/730| 2300/610| 0.95   | 0.13  |
| E. coli           | 16        | 2/15.6   | 10/760 | 540/530 | 1.99   | 0.25  |
| S. salivarius     | 6         | 1/3.3    | 810/735| 1176/610| 2.52   | 0.21  |
| S. pyogenes       | 5         | 1/3.2    | 135/745| 728/600 | 5.57   | 0.13  |
| E. aerogenes      | 24        | 3/58.0   | 196/745| 1353/600| 2.67   | 0.15  |
| K. pneumoniae     | 15        | 3/29.9   | 9/760  | 448/535 | 15.4   | 3.41  |
| S. sonnei         | 25        | 5/40.0   | 405/745| 1922/600| 1.71   | .04   |
| S. aureus         | 6         | 1/4.2    | 55/760 | 3240/540| 1.11   | 0.07  |

* Base: The base of the peak in millimeters produced by a 580-nm scan of a microbial colony photograph (see Fig. 6A).
* Alpha: First number represents the number of transmission peaks obtained on photoanalysis (refer to Fig. 6A), the slant has no mathematical meaning, whereas the second number equals the base times the area of the peak divided by 100.
† Peaks 1 and 2 refer to the transmission recording peaks (see Fig. 6 and 7B).
‡ SRU: static infrared unit; λ: wavelength of peak transmission.
‡ Beta: a value derived according to the formula on page 209.
‡ SD, standard deviation of beta from five experimental measurements.

DISCUSSION

The data presented in this study have demonstrated that bacterial colonies, even of closely related species, possess sufficient structural and compositional differences to provide characteristic IR interactions. These characteristics can be recorded on IR-sensitive film. Subsequent spectrophotometric analysis of the film provides data useful in quantitating specific differences. The colony’s infrared transmission response suggests a dependence on the collective effect of colony density, color, geometry, and chemical composition. IR transmitted through the bacterial colonies has the capacity to reduce dyes in the film which are IR wavelength specific. The reduction of these selective dyes is characteristic of the colony through which the IR radiation has passed.

The data presented in this paper are easily obtained, require limited specialized equipment, and may be feasible in a clinical as well as research setting. Control experiments using white light and noninfrared Ektachrome film have failed to produce similar geometric data or light interaction data which could allow bacterial identification (Fig. 9).

The overall effect of the inherent bacterial IR emissions (passive infrared) incident on the film is difficult to appraise. E. coli produces about $1.4 \times 10^{-6}$ W of IR per s per colony area when ideal emissivity conditions exist (1, 3), whereas Staphylococcus aureus produces only about $9.0 \times 10^{-6}$ W of IR per colony area per s. However, currently available IR film requires a minimum of about $3.0 \times 10^{-6}$ W of IR per colony area per s, in order for the film to be exposed sufficiently to be detected by photoanalysis (4, 5). Therefore, the inherent IR of the colonies is not
sufficient to effect a response on the film due to their low power and the long wavelength of such passive emissions. These limitations in detection of passive bacterial IR require that a carrier infrared source be supplied, such as the photomicrographic system used in these studies. Due to the low sensitivity of the film and short IR wavelength required for exposure, it is doubtful that the small but characteristic amount of passive infrared emission from the bacterial colonies contributes significantly to the overall transmission response of the test organisms.

The data contained in Table 1 do not suggest that any interpolations can be made regarding specific information on chemical composition of the cells. Variations in the system such as film emulsion changes, changes in film developing chemicals, compositional changes in bacterial growth media, and different microphotographic systems prevent the data in Table 1 from being established as standard values for the organisms shown. With future refinement and standardization of media and film development, it may well be possible to establish a reference by which unknown organisms could be identified by using this infrared transmission procedure.

ACKNOWLEDGMENT
This investigation was supported in part by the Office of Naval Research Contract N00014-70-0074-003.

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