The Use of Computerized Television Morphodensitometry in the Study of Microbial Antagonism

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UDC 615.33.331:579.8:681.2

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 118, № 7, pp. 63-66, July, 1994
Original article submitted January 8, 1994

Computerized television morphodensitometry has been applied for the first time to evaluate quantitatively and qualitatively the inhibitory effects associated with microbial antagonism. It is shown that the use of CTM for this purpose can add to the existing knowledge about the inhibitory potentials of biologically active substances which microbial antagonists produce, in particular by demonstrating that when no growth of bacterial test cultures exposed to an antagonist is detectable visually there may still be some growth in the form of L transformation, and by enabling this growth to be quantified.

Key Words: *morphodensitometry; image analysis; microbial antagonism*

To date, systems for pattern recognition and image analysis have been used in microbiology very rarely and mostly in primitive routine studies (e.g., to follow the growth of yeasts or variations in bacterial cell numbers in a substrate [4,7-10]. This is because a large gap exists between the potentials of morphodensitometric systems and their practical output. Yet computerized television morphodensitometry (CTM) — a method by which morphological characteristics of biological specimens can be reconstructed from their optical densities using digital image processing — not only expedites quantitative analyses but also yields important and qualitatively new information [2] that remains virtually inaccessible to visual observations and to conventional microbiological techniques (such as light or electron microscopy) because of the enormous natural variability of microbiological objects and of their morphological structures.

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Methods of determining microbial antagonistic activity that rely on the use of diffusion into agar [5] can provide only visual information about the actions of biologically active substances (BAS) produced by the microorganisms and do not permit a quantitative evaluation of cell death in the test cultures exposed to the antagonist: the visually observed growth retardation or cessation does not yet give an indication of the actual inhibitory antagonistic effect as no account is taken of bacterial L transformation and reversion. The use of ATB densitometers (Switzerland) to determine the sensitivity of enterobacteria to antibiotics [6] failed to provide data on morphological changes in bacterial populations and, moreover, a comparison with the traditional disk method showed significant differences between the results produced by these two methods.

In view of the foregoing, we decided to adapt the method of CTM for quantitative evaluation of microbial antagonism, using model test cultures of *Staphylococcus aureus* and *Escherichia coli.*

MATERIALS AND METHODS

The test cultures used were the *E. coli* strains K12, 09, 0101, 099, and 04 and the *S. aureus*

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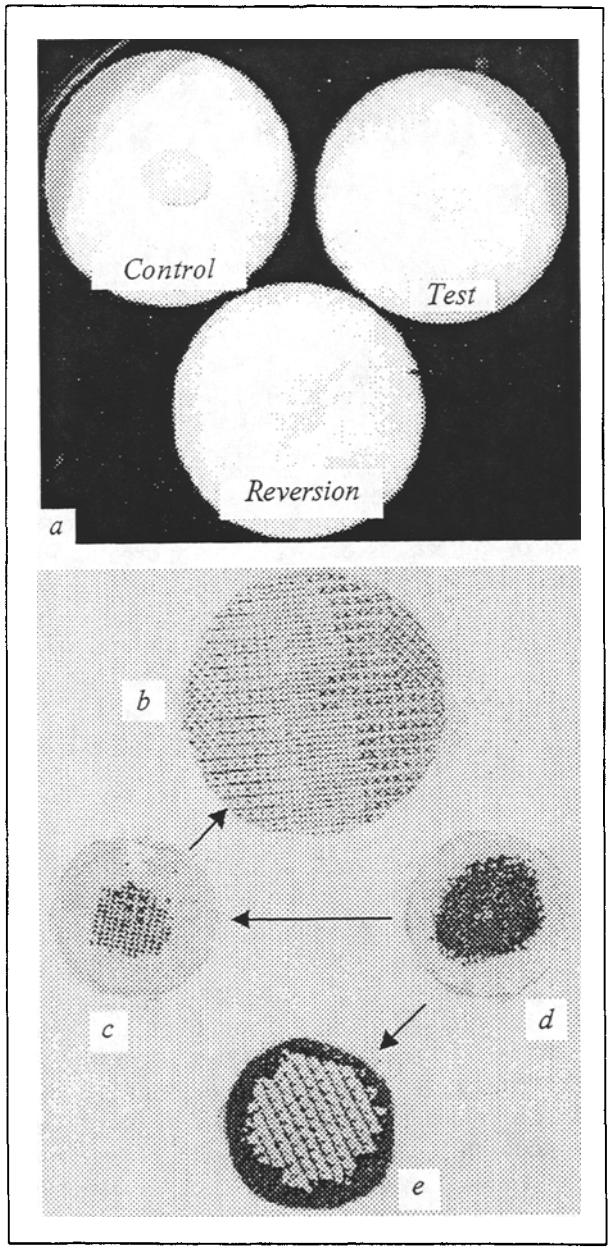

Fig. 1. Impact of a BAS producer on test cultures and computer processing of their images. *a*) growth of cultures on filters in the form of a spot; *b*) image obtained with visually imperceptible colonies; *c*, *d*, and *e*) sequential "isolation" of the spot and its enhanced visualization through discrimination, segmentation, and zooming ("electronic magnification").

strain 209-R. BAS-producer cultures were *Lactobacillus fermenti*, *Lactobacillus plantarum*, and the *Kolir* preparation. All these strains possessed typical cultural and biochemical properties.

Microbial antagonism was detected with a procedure [1,3] which we had improved and adapted for instrumental quantitative and qualitative studies of bacteria. Briefly, a membrane filter (*Vladipor*, No. 2, 4, or 5) was placed on the surface of a dense nutrient medium (MPA, MRS-1, MRS-5, or a specialized medium for L forms), and 0.5 μl of a producer culture (10^7 or 10^8 bacterial cells) in 0.7% Difco agar was applied to the filter (one drop). After incubation, the filters together with their grown cultures were removed and replaced with new filters to which the appropriate test cultures were then applied in a strictly defined amount, followed by incubation under predetermined conditions. The growth of the bacterial cultures proceeded in the form of a spot within the limits of the drop. The results were recorded both morphodensitometrically (measuring the area and the mean and integral optical density of the spots) and with scanning electron microscopy (morphological evaluation).

The morphology of the bacterial colonies was evaluated by examining them in a Hitachi-800 electron microscope with an E-8010 scanning attachment (Japan) after fixation in glutaraldehyde and dehydration in alcohols of ascending concentrations. Sputtering was done with gold on an E-109 sputterer.

The quantitative evaluation of the inhibition of test cultures by the antagonist-produced BAS was carried out by the method of CTM, for which the DIAMORF computerized television image analysis system and the DIAMORFO software package were employed, both designed at the Institute for Physicochemical Medicine and relying on the use of an IBAS-2 facility (Opton, Germany).

RESULTS

The BAS produced by the antagonists inhibited cell growth in the test cultures to varying degrees. The degree of inhibition could be assessed subjectively (visually) by noting the decrease in the size of the spots, which in some cases disappeared completely, as is exemplified in Fig. 1, *a*. For an objective evaluation of the inhibition, CTM was used, by which the inhibitory effect of the antagonist can be quantified precisely by measuring the area and optical density of the spot on their images. For this evaluation, the areas and optical densities of the spots containing bacterial colonies were at first measured on spot images using control preparations (Fig. 1, *a*). After the test strains had been exposed to their microbial antagonists, the spots were barely discernible or invisible. With CTM, however, it was possible to obtain positive results for preparations of extremely low contrast whose visual assessment yielded negative results (Fig. 1, *b*). Thus, in cases where no spots with inhibited growth of test cultures were visible on computer printouts, optical characteristics of such spots could still be recorded from their individual

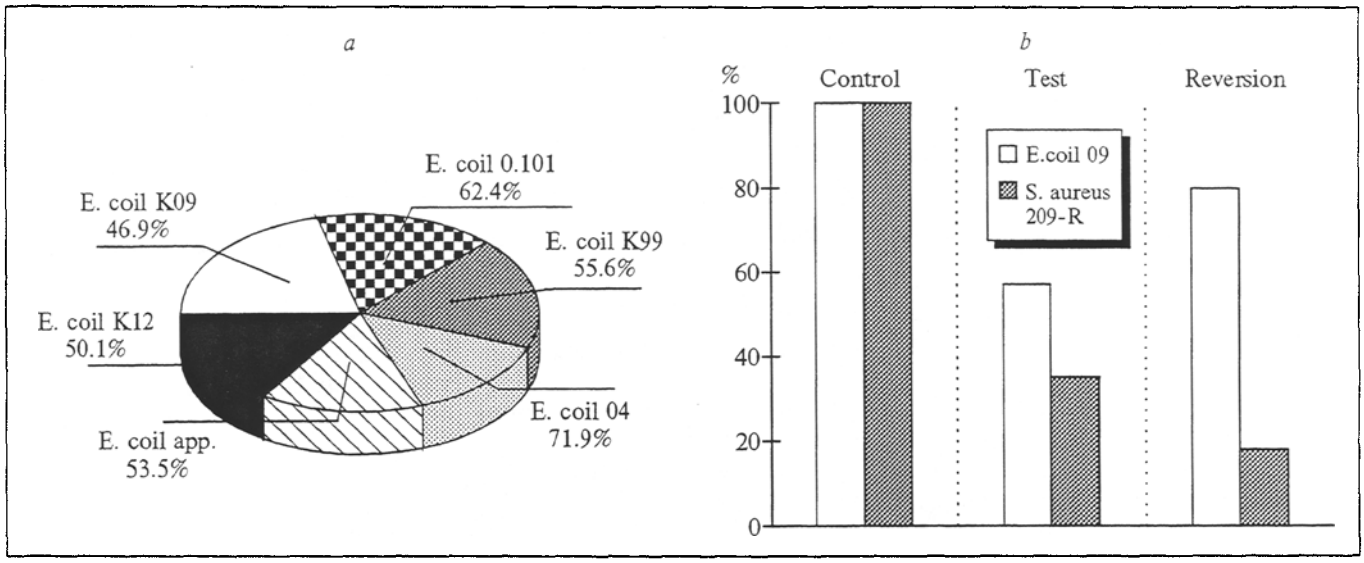

Fig. 2. Use of computerized television morphodensitometry for quantitative evaluation of the impact exerted by the *Kolir* preparation on test cultures. *a*) various *E. coli* strains; *b*) reversion of test cultures.

images obtained through discrimination, segmentation, and zooming (Fig. 1, *c*, *d*, and *e*).

The *Kolir* preparation reduced the populations of *E. coli* strains by 32% on average (Fig. 2, *a*). The membrane filters in which the growth of *E. coli* K12 colonies was strongly inhibited by the BAS of the *Kolir* preparation were placed on the specialized nutrient medium for L forms. After a 24-h incubation in a thermostat at 37°C, it was very difficult to detect the growth of these colonies visually. Using CTM, however, it was found that the reversion of *E. coli* K12 cells amounted to only 17.1% whereas the reversion of *S. aureus* cells under the same conditions was about 90% (Fig. 2, *b*).

In the control preparations, *S. aureus*, *E. coli*, and *Lactobacillus* cells all had a typical morphology (Fig. 3, *a*, *b*, and *c*). The populations of these cells were homogenous. Staphylococci had spherical shapes, and their association could be observed (Fig. 3, *a*); *E. coli* cells appeared as short rods, were

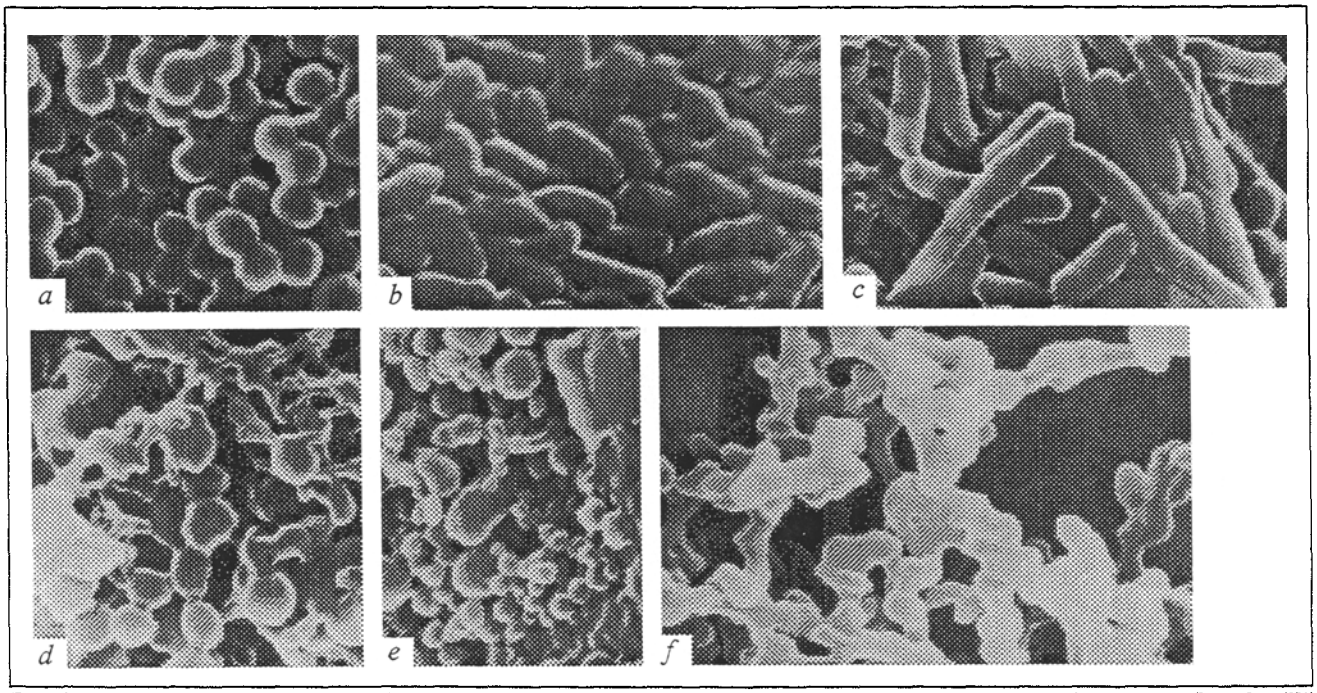

Fig. 3. Use of scanning electron microscopy for qualitative evaluation of the impact exerted by a BAS producer (lactobacilli) on test cultures. ×12,000. *a*, *b*, and *c*) intact *S. aureus*, *E. coli* (test strains), and *L. plantarum* (antagonist) cultures, respectively; *d*, *e*, and *f*) impact of the BAS on test cultures: heteromorphous cell growth in a staphylococcal microcolony, with L transformation (*d*) and reversion (*e*); *f*) heteromorphous cell growth in an *E. coli* microcolony.

TABLE 1. Results of the Quantitative Analysis of the Action of *Lactobacillus*-Prodused Biologically Active Substances on Cell Populations of the Test *S. aureus* Strain.

Bacterial culture	Morphodensitometric parameters			Proportion of cells in the population, %
	Area	OD	IOD	
Control *S. aureus* organisms	813	6221	505.8	100
Test *S. aureus* organisms	397	2176	86.39	17

Note. OD = optical density; IOD = integral optical density.

arranged in an orderly manner, and were also in association (Fig. 3, *b*); lactobacilli, too, were rod-shaped and densely packed (Fig. 3, *c*).

Of special interest appears to be the antagonistic action of lactobacilli against staphylococci (Fig. 3, *d*). Examination of those areas of membrane filters where no staphylococcal colony growth was detectable visually revealed a heteromorphous growth of staphylococcal cells characteristic of their L transformation. Thus, deformed cells of various sizes carrying very small spherical cells on their surface were seen, as were areas containing small tortuous rod-shaped revertant cells (Fig. 3, *e*).

The BAS produced by lactobacteria decreased the staphylococcal cell population by 83% (Table 1).

Heteromorphous growth was also induced by the *Kolir* preparation in *E. coli* populations; altered cells of spheroplast and protoplast types were seen (Fig. 3, *f*).

Thus, our method for the study of microbial antagonism has allowed CTM to be used for the first time to quantify inhibitory effects associated with this phenomenon. The use of CTM for this purpose adds to the existing knowledge about the inhibitory potentials of bacteria-produced BAS by showing that where no bacterial growth is detectable visually there may actually be some growth in the form of L transformation and by enabling the degree and stability of the latter to be evaluated in quantitative terms.

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