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Effect of physiological heterogeneity of *E. coli* population on antibiotic susceptivity test

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According to the instantaneous growth rate (dN/dt) of *E. coli* CVCC249 growing in batch culture, the entire growth progress was distinguished into four phases: accelerating growth phase, constant growth phase, decelerating growth phase and declining phase, in each of which obvious variation in physiological and biochemical properties was detected, including total DNA, total protein, and MTT-dehydrogenase activity, etc., that led to difference in their antibiotic susceptivity. Antibiotic susceptivity of the population sampled from each phase was tested by Concentration-killing Curve (CKC) approach following the formula $N=N_0/\{1+\exp[r\cdot(x-BC_{50})]\}$, showing as normal distribution at the individual cell level for an internal population, in which the median bactericidal concentration BC_{50} represents the mean level of susceptivity, while the bactericidal span $BC_{1\text{-}99}=(2\ln N_0)/r$ indicates the variation degree of the antibiotic susceptivity. Furthermore, tested by CKC approach, the antibiotic susceptivity of *E. coli* CVCC249 population in each physiological phase to gentamicin or enoxacin was various: susceptivity of the population in the constant growth phase and declining phase all increased compared with that in the accelerating growth phase for gentamicin but declined for enoxacin. The primary investigations revealed that the physiological phase should be taken into account in the context of antibiotic susceptivity and research into antimicrobial mechanism. However there are few reports concerned with this study. Further research using different kinds of antibiotics with synchronized continuous culture of different bacterial strains is required.

instantaneous growth rate, batch culture, physiological heterogeneity, concentration-killing curve, antibiotic susceptivity test

It will be more available for antibiotic therapy to test the antibiotic susceptivity for certain pathogenic-bacterial population when simulating the condition of *in vivo* than use of some standards *in vitro*, even that of the Clinical and Laboratory Standards Institute. In fact there are many factors that can affect the result of antibiotic therapy such as antibiotic pharmacokinetics concentration, incubation condition, the cell number and physiological phase of inocula, etc[1]. The first three factors are firmly normalized in antibiotic susceptivity test (AST) and the pharmacodynamics can be described with Concentration-Killing Curve (CKC)[2,3], but by now the effect of physiological phase, especially physiological heterogeneity, has long been underestimated.

It is well known that bacteria procure much higher resistance to antibiotics than normal cells when they form into spore, gemma, biofilm or L-form. Furthermore, every bacterial cell will undergo life-cycle with a series of changes in morphology, physiological and biochemical properties[4,5]. In this life cycle, the cells become into a population, that are inevitably heterogeneous in cell morphology, physiology and genetics, even though they might come from a single cell. For example, in the

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batch culturing population, there is a gradual change in the numbers of viable cells in division-cycle, viable but non-dividing cells, dead cells and even autolyzing cells[6−10].

In the regular proliferation, the population will increase exponentially, leading to the rapid change of culture environments and then, as a result, these changes further affect the reactive rate of a series of enzyme-catalyzed reaction[11−14]. All the changes in physiological and biochemical properties of the population will result in antibiotic susceptivity diversity, which is significant for clinical application of antibiotics in different phases of the infection.

In the previous study, we proposed the Spline-Numerical-Gaussian (SNG) method according to instantaneous rate (V_{inst}), and successfully distinguished the entire growth curve of *E. coli* batch culture into four phases: accelerating growth phase, constant growth phase, decelerating growth phase, and declining phase[6,7]. Following this confirmed result, the CKC approach, established recently in our laboratory, was used to determine the dynamic antibiotic susceptivity of *E. coli* population sampled from different growth phases. This investigation is expected to provide some new information for antibiotic susceptivity test and guiding clinical therapy.

1 Materials and methods

1.1 Strain

E. coli CVCC 249 was kindly provided by the Veterinary Microbiological Cultures Collection Center in China. It is noticeable that this bacterium can infect domestic chickens.

1.2 Distinguishing growth phases of *E. coli* CVCC249 in batch culture based on instantaneous rate

E. coli CVCC249 was purified by a series of repeated isolation until colony formation on MBC plates was abolished. After 12 h of shaking incubation at 37℃, 200 rpm, the culture was centrifuged for two times and diluted with sterile physiological salt solution, and maintained at 4℃ to obtain rest cells as uniform inoculums of about 1.8×10^9 CFU/mL.

About 2×10^7 rest cells of *E. coli* CVCC249 were inoculated into a fermentor containing 5 L Luria-Bertani (LB) medium and allowed to incubate at temperature of 37℃, shaking rate of 200 rpm, and air-ventilating rate of 3 mL/min. Culture samples were collected at 1 h intervals. The samples of bacterial culture were centrifuged and diluted with physiological salt solution to the initial volume. Then the turbidity, total protein, total nucleic acid and MTT-dehydrogenase activity were determined by spectrophotometry at wavelength of 600, 280, 260 and 510 nm, respectively. The entire growth curves were constructed by plotting the turbidity (N) vs. time (t), and SNG method was used to distinguish the growth phases based on V_{inst}[6,7].

1.3 The antibiotic susceptivity of batch culturing population in each growth phase determined by CKC approach

Each of 10 mL bacterial suspension was sampled at 1, (5, 7), 12 and 24 h from the above batch culture that corresponded to the four different growth phases, respectively. After opportune dilution, 20 μL suspension of each sample containing 500−1000 cells was spread over the surface of the LB-agar plate mixing with a monotonic gradient concentration of enoxacin or gentamicin. This experiment was designed on such an assumption: the cell number of inoculums (500−1000) is much less than the base population of spontaneous mutation, and there is little probability of the presence of the spontaneous mutants in those inoculums, so as to ensure the genetic homogeneity of the inoculum. Therefore the colony occurring on the above LB-plate during incubation should be only caused by effects of antibiotics. In general, the plates were incubated at 37℃ for 24 h until the number of visible colonies per plate, i.e., the number of CFU per plate, was counted. Then, CKC were constructed by plotting the number of CFU per plate (N) vs. the concentration (x), and equation $N=\frac{N_0}{1+e^{r(x-BC_{50})}}$ was used to fit the sigmoid CKC with Graph Pad Prism (version 4.0) software. In the equation, N_0 is the initial cell number of inoculums (500−1000), BC_{50} is the median bactericidal concentration and r is bactericidal intensity.

2 Results

2.1 Distinguishing growth phases of *E. coli* CVCC249 in batch culture based on instantaneous rate

As shown in Figure 1, during the entire growth progress, the kinetic changes of turbidity, total protein, total nu-

cleic acid and MTT-dehydrogenase activity vs. time were all quite accordant. According to the change of V_{inst} (dN/dt) of turbidity, the entire growth progress was distinguished into four phases: accelerating growth phase (A), constant growth phase (B), decelerating growth phase (C) and declining phase (D). As detected by flow cytometry, the ratio of live cells/dead cells in each growth phase was quite different with each other and was of high positive correlation with V_{inst}[7]. This result demonstrated that division of the growth phases based on SNG method was more significant for comparing the antibiotic susceptivity of bacterial population growing in different physiological phases, since it focused live cells as target of AST.

2.2 Comparison of individual antibiotic susceptivity of an internal bacterial population exposed to different antibiotic concentration

The susceptivity of population in each phase to enoxacin or gentamicin was determined by CKC approach. The dynamic change of CFU/plate vs. concentration described by CKC method reflected the variation of individual antibiotic susceptivity within a bacterial population of genetic homogeneity[3,15], and quantified the rule with parameters BC_{50}, $BC_{99} = BC_{50} - \frac{\ln N_0}{r}$, $BC_1 = BC_{50} + \frac{\ln N_0}{r}$ (Figure 2). Just like the growth curve describing the reproduction and decline of live cells, CKC approach can characterize the dynamics death of live cells. Killing rate (dN/dx) was the killed cell number per concentration unit of drug. It reached the max $-rN_0/4$ at concentration of BC_{50} and dropped off when concentration tended to each side $(BC_{50} \pm \frac{\ln N_0}{r})$, belonging to normal distribution. The bactericidal concentration span $BC_{1-99} = \frac{2}{r}\ln N_0$ from BC_{99} to BC_1 indicated the degree of antibiotic susceptivity heterogeneity in a batch culture population.

2.3 The variation of antibiotic susceptivity of a bacterial population in different growth phases

Under batch culture condition, a bacterial population may be heterogeneous in many important characteristics, such as live/dead cell rate, cell shape and size, DNA and special mRNA content per cell and MTT activity. This heterogeneity decided the growth curve of a population[6−14, 16], and also decided CKC of population in different growth phases as shown in the present study (Figures 3 and 4). Using the accelerating growth phase population as control, constant growth phase and de-

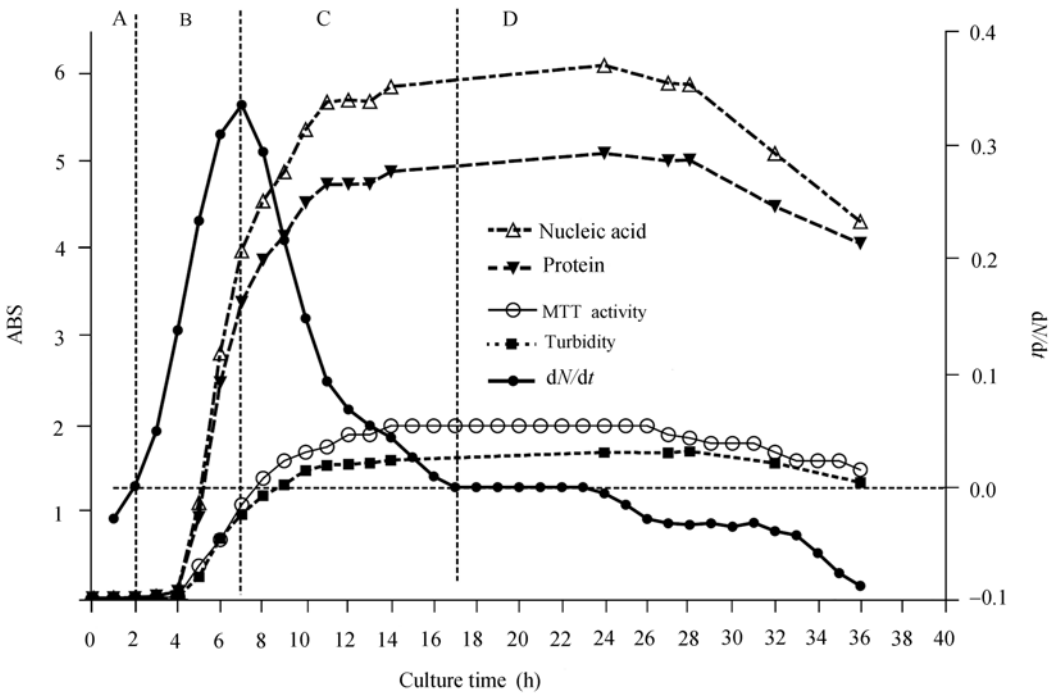

Figure 1 The kinetic changes of turbidity, total protein, total nucleic acid and MTT-dehydrogenase activity vs. time during the entire growth progress of batch culturing bacterial population and four phases distinguished with instantaneous rate. (A) Accelerating growth phase, (B) constant growth phase, (C) decelerating growth phase, and (D) declining phase.

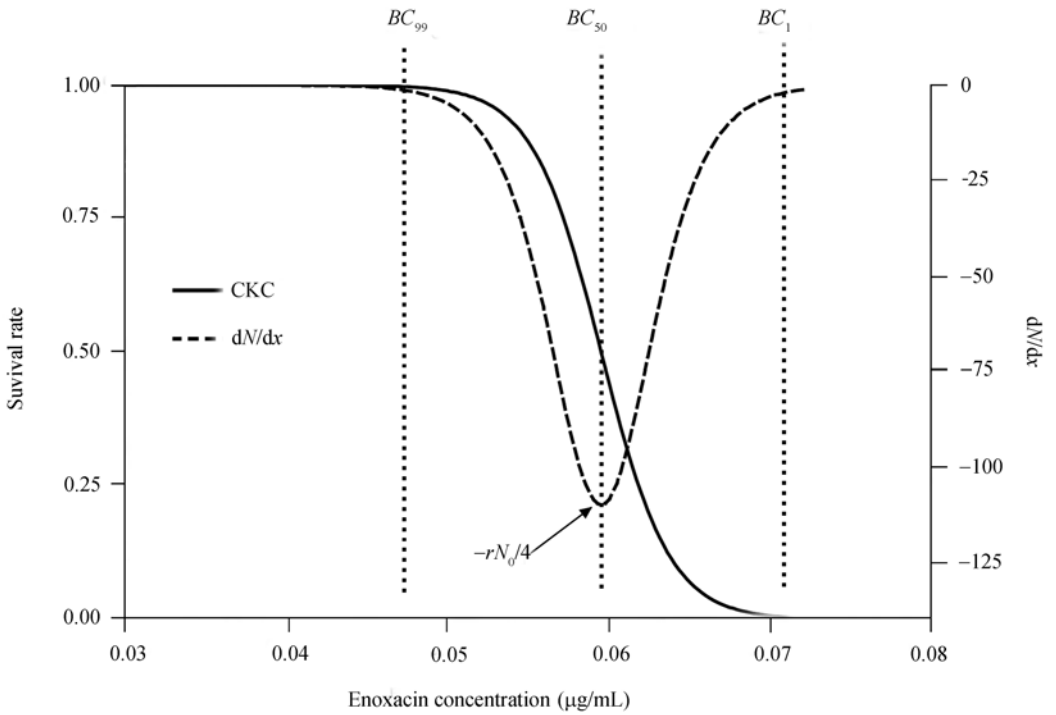

Figure 2 Concentration-killing curve of *E. coli* to enoxacin and its killing rate curve (dN/dx). BC_1 is the bactericidal concentration that only one cell survives, and BC_{99} is the bactericidal concentration that only one cell is killed. The bactericidal concentration span $BC_{1-99}=\frac{2}{r}\ln N_0$ from BC_{99} to BC_1 indicated the degree of antibiotic susceptivity heterogeneity in a batch culture population.

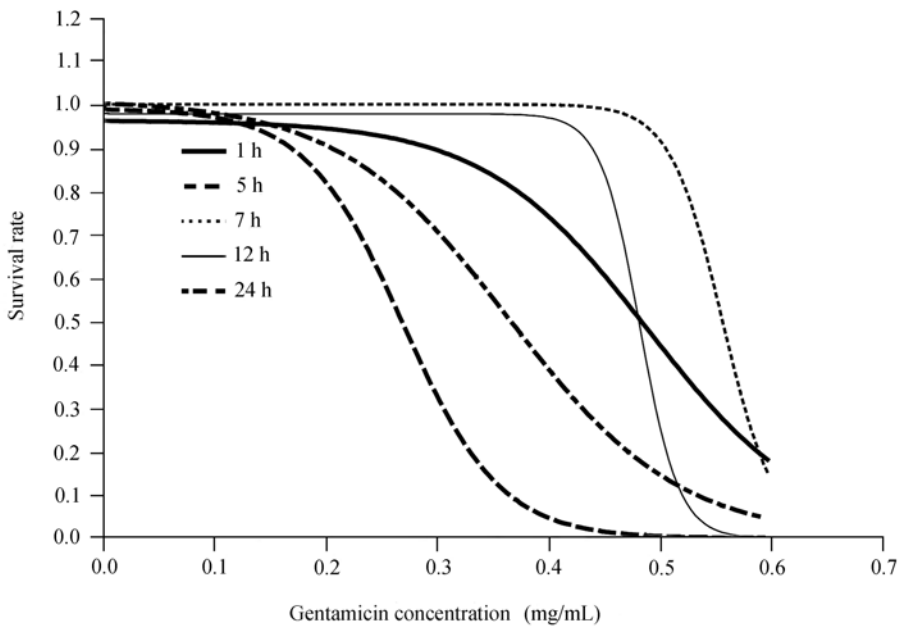

Figure 3 Concentration-killing curve of *E. coli* CVCC249 population in different growth phases to gentamicin.

clining phase population were more susceptive, and decelerating growth phase population was less susceptive for gentamicin; however, for enoxacin, only constant growth phase population was less susceptive, and no notable change happened to other phases population. This phenomenon cannot be revealed with routine AST.

3 Discussion

3.1 The physiological state of a bacterial population is one of important factors to AST

AST is the important basis and dependence for studying

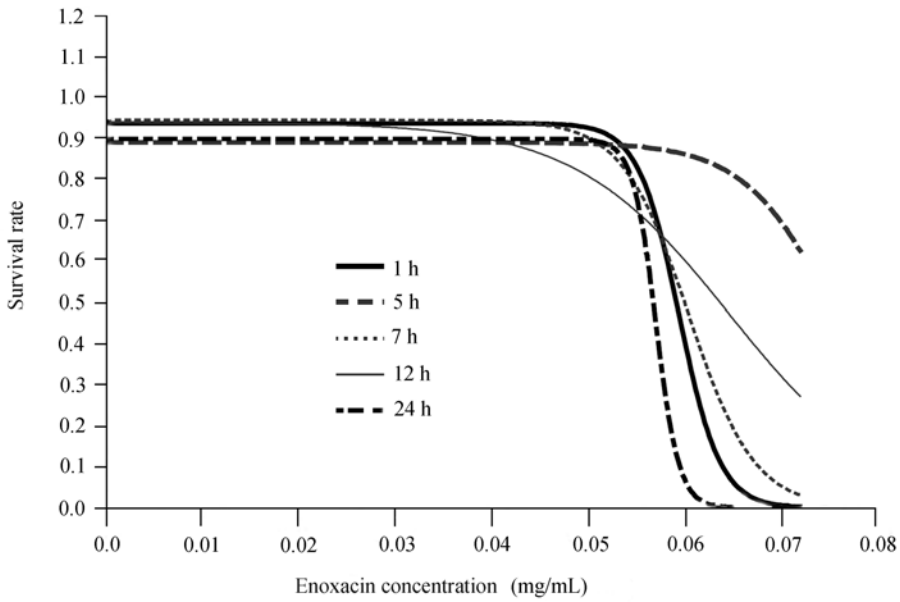

Figure 4 Concentration-killing curve of *E. coli* CVCC249 population in different growth phases to enoxacin.

antibacterial or bactericidal mechanism and guiding clinical therapy. When a growing bacterial population is exposed to one antibiotic, there will be a competition between growth and killing depending on the kind and concentration of antibiotic and the bacterial culture conditions. Antibiotic can trigger the bacterial apoptosis, and on the other side, the bacteria try to survive through any way involving genetic mutation and physiological adaptation[17−21]. However, in the general AST methods, the rest cells or over-night-culture is selected as assay inoculum, so the effect of different growth phases on AST is indistinct and underestimated. In the present study, with the combination of SNG model for growth phase division of batch culture population and CKC method for dynamic analysis of bactericidal potency, the effect of physiological state of inoculum to AST is expressly revealed.

3.2 The physiological state of a bacterial population is decided by individual cells in different growth phases, and antibiotic susceptivity variation of it can be estimated by CKC

Antibiotic susceptivity of a bacterial population lies, biochemically, on many enzyme-catalyzed activities relating to bacterial growth, division, DNA replication and the special inhibition to these enzymes. The live cells/dead cells ratio and changes in cell dimension of batch culture population detected by laser confocal microscopy and flow cytometry clearly indicated the physiological heterogeneity of genetically homogenous population even derived from a single cell[4,7,22]. However, antibiotic susceptivity of single cell cannot be detected by now. CKC method is such a dynamic model that can characterize macroscopically the antibiotic susceptivity variation of a population of 500−1000 cells which had no chance to mutate or have mutant. Along with the antibiotic concentration arising, the more susceptive cells were killed, and the most cells were killed at BC_{50}, then the less susceptive cells were killed until the last one, at the killing rate belonging to normal distribution $(BC_{50} \pm \frac{\ln N_0}{r})$ [3].

The internal heterogeneity of a population, changing along with the incubation, not only influenced the result of AST of different population, but also resulted in different rules for different antibiotics. The bactericidal gentamicin inhibited the synthesis of protein by joining 30S ribosome, so the rest cells were easy to be inhibited when they started synthesis of RNA and proteins in the accelerating growth phase, and the less of the synchronization, the stronger the heterogeneity; the population in the constant growth or decelerating growth phase was more resistant to gentamicin for they had stored considerable protein, and the heterogeneity was lower; however, the population in declining phases was easy to be inhibited again when their protein was depleted and some cells were dying, and heterogeneity became stronger. Enoxacin inhibits bacteria growth from headstream-DNA gyrase so as to stop DNA replication, transcription and repair, therefore, the heterogeneity and

susceptivity variation was not obvious for each phase except for the accelerating growth phase when DNA synthesization was strong. For the similar reason, it is easy to explain the stronger inhibition of penicillin to population in the exponential growth phase.

This paper reveals the notable influence of physiological heterogeneity on the result of AST only through the experimental system of gentamicin and enoxacin vs. *E. coli* CVCC249. Further research by using different kinds of antibiotics with synchronized continuous culture will be required for in-depth analysis of biochemical mechanism to sum the universal law.

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