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ORIGINAL ARTICLE

Bacteriostatic versus bactericidal activity of ciprofloxacin in *Escherichia coli* assessed by flow cytometry using a novel far-red dye

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As common microbiological methods for the assessment of bacteriostatic or bactericidal activities are very time-consuming, in this work we describe that the use of a novel far-red fluorescent stain, Vybrant DyeCycle Ruby (DCR) for the flow cytometric analysis of fluoroquinolone (ciprofloxacin) bacteriostatic and bactericidal activities in *Escherichia coli* proved to be specific for bacterial DNA and, after ciprofloxacin exposure, DNA distribution analysis was achieved using a 7.5 μM DCR concentration to stain 5×10^5 ethanol-fixed bacterial cells. The analysis of the bacterial DNA histograms obtained from the ciprofloxacin concentrations tested, enabled the distinction between ciprofloxacin bacteriostatic and bactericidal activities.
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INTRODUCTION

In clinical practice, optimal dosing is a critical component of antibiotic therapy, regardless of the type or class of agents employed.[1] In patients with serious bacterial infections, the use of maximum tolerable antibiotic doses improves therapeutic outcomes, and may prevent the development of resistance.[2] Bactericidal rather than bacteriostatic agents may be preferred, as these agents may limit the development of resistance.[3] However, even when bactericidal agents are used, the administered dose must be sufficient to achieve bactericidal activity,[1] because with some antibacterials (for example, aminoglycosides and fluoroquinolones) bacterial killing is concentration-dependent. Therefore, it is important to develop fast and simple methodologies to assess whether the antibiotic dose administered produces bactericidal or bacteriostatic activities against the pathogens.

The use of flow cytometry as an important tool in bacteriology is emerging.[4] Flow cytometric measurement of DNA content may be important in understanding and quantifying bacterial heterogeneity. Many studies involving bacterial DNA content analysis have been performed with UV-excitable, DNA-specific fluorescent stains, such as Hoechst dyes and 4′,6-diamidino-2-phenylindole (DAPI). However, the use of these fluorescent stains required the use of UV lasers, and the flow cytometers that can accommodate UV excitation remain uncommon and are considerably more expensive than conventional cytometers.[5] In order to circumvent this, new fluorescent stains excitable with violet (395–415 nm), blue (488 nm) and red (635 nm) lasers have been developed. Examples of such fluorescent stains are the Vybrant DyeCycle series (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and DRAQ5 (Biostatus Limited, Leicestershire, UK). Some of these dyes have been successfully used to assess intracellular DNA distribution in live[6–8] and fixed cells.[9] Recently, a Vybrant DyeCycle stain was developed, Vybrant DyeCycle Ruby (DCR), which has the same features of the other related stains and can be used with any excitation source from 488 to 690 nm with fluorescence emission in the near-infrared region.

Fluoroquinolones are broad-spectrum antibacterial agents that are extensively used for treatment of infections.[10] Depending on their concentration, fluoroquinolones may exhibit bacteriostatic or bactericidal activities.[11] For instance, ciprofloxacin, a fluoroquinolone, exhibits a bacteriostatic activity when the replication of DNA is inhibited by inhibiting DNA gyrase and a bactericidal activity caused by bacterial DNA fragmentation. Therefore, this work reports the applicability of a novel far-red dye, DCR, for the rapid and easy evaluation of bactericidal and bacteriostatic activities of ciprofloxacin. This analysis is performed by the evaluation of the dye effects in the DNA content of *E. coli* by flow cytometry.

MATERIALS AND METHODS

Bacterial strains and antibiotics

The bacterial strain used was *Escherichia coli* ATCC 25922. Ciprofloxacin (Sigma-Aldrich, St Louis, MO, USA) stock solutions were prepared at 1 mg ml^{-1} in 0.1 N HCl, sterilized by filtration (0.22 μm pore-size filter), dispensed in 0.5 ml aliquots and frozen at −80 °C until use.

Ciprofloxacin susceptibility testing

The *in vitro* activity of ciprofloxacin was determined by the broth microdilution method, according to the Clinical and Laboratory Standards Institute

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(M7-A6),[12] with cation-adjusted Müeller–Hinton broth. Microtiter plates containing serial dilutions of ciprofloxacin ranging from 4 to 0.001 μg ml^{-1} were inoculated with *E. coli* to yield approximately 5×10^5 cells per ml in a 100 μl final volume. The plates were incubated for 18 h at 37 °C. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of ciprofloxacin that prevented visible growth.

Time-kill studies

Time-kill assays were performed by the broth macrodilution method, according to the Clinical and Laboratory Standards Institute guidelines.[13] For the concentration-dependent killing curve studies, various multiples of the MICs (0.5, 1, 2 and 4) were used to detect differences in killing. Shake flasks (250 ml) containing 50 ml of cation-adjusted Müeller–Hinton broth with the appropriate ciprofloxacin concentrations were inoculated with 50 ml of the exponentially grown *E. coli* cells, to yield a final concentration of approximately 1×10^6 cells per ml. Flasks were incubated at 37 °C, and aliquots were removed at 0, 2, 4, 6 and 24 h for the determination of viable counts. Serial dilutions were prepared in sterile 0.85% sodium chloride solution and plated according to the method previously described.[14] The plates were incubated at 37°C for 18 to 22 h, and the number of colonies was determined. The detection level by this plating method was 1×10^2 CFU ml^{-1}.

Killing curves were constructed by plotting the $\log_{10}$ CFU ml^{-1} versus time over 24 h, and the change in bacterial concentration was determined. Bactericidal activity was defined as a reduction of 99.9% ($\geqslant$3 log10) of the total number of CFU ml^{-1} in the original inoculum.[15] Bacteriostatic activity was defined as maintenance of the original inoculum concentration or a reduction of less than 99.9% ($<$3 log10) of the total number of CFU ml^{-1} in the original inoculum.[16]

Ciprofloxacin exposure and cell recovery for flow cytometric and microscopic analysis

A pre-culture was performed by growing *E. coli* cells in Brain Heart Infusion broth until mid-exponential phase of growth was reached. Cells taken from the pre-culture (1×10^6 cells per ml) were incubated in cation-adjusted Müeller–Hinton broth with ciprofloxacin at 0.5, 1, 2 and 4 times the MIC value. As a control of bacterial growth, bacterial cultures were grown without the addition of ciprofloxacin. All cultures were incubated in the same conditions described previously for the time-kill studies. Three 1-ml samples were removed from the control culture prior to incubation, and further three 1-ml samples were removed from cultures after 2, 4 and 6 h of incubation. Samples were centrifuged at 10,000 *g* and the pellet was washed with phosphate-buffered saline (PBS) buffer (pH 7.4) once and resuspended in 2 ml Müeller–Hinton broth for plate counts and microscopic analysis. In order to improve the ability to resolve ploidy, cell pellet was also resuspended in Müeller–Hinton broth containing 300 μg ml^{-1} rifampicin and 10 μg ml^{-1} cephalexin, as previously described.[9] After this treatment, bacterial cells were ethanol-fixed as described below.

Bacterial fixation and staining

A 700 μl volume of ice-cold absolute ethanol was rapidly added to the 300 μl of recovered cells after ciprofloxacin exposure, as described above. This suspension was mixed by rapid pipetting to prevent cell clumping. The samples were then incubated at 4 °C for 30 min for fixation. Fixed cells were stored at −20 °C until they were stained, and analyzed on the flow cytometer. After optimization of both incubation time and DCR concentration, 5×10^5 cells per ml were washed once with PBS buffer (pH 7.4), incubated with 7.5 μM DCR in PBS buffer (pH 7.4) at 37 °C for 30 min in the dark and washed once in the same buffer before the flow cytometric analysis.

As a control for DCR bacterial DNA specificity, ethanol-fixed cells were incubated with DNase and/or RNase as described.[9]

Flow cytometry

DCR (Molecular Probes, Invitrogen, part of Life Technologies, Carlsbad, CA, USA) stock solution (2.5 mM) in dimethylsulphoxide was stored at −20 °C. Bacterial samples were analyzed on a BD Biosciences FACSCalibur (BD Biosciences, San Jose, CA, USA). Acquisition was performed with CellQuest Pro software (BD Biosciences) and based on light-scatter and fluorescence signals resulting from 15 mW laser illumination at 488 and 635 nm. Light-scatter measurements were acquired logarithmically, while fluorescence signals were acquired linearly. DCR fluorescence signal was screened with a FL-4 (661 nm) bandpass filter. Threshold levels were set on SSC to eliminate noise or particles (of cellular debris) much smaller than intact cells. Bacterial cells were gated according to FSC/SSC parameters. Daily calibration was performed using CaliBRITE beads (BD Biosciences), according to the manufacturer instructions. Sample acquisition was operated at low flow rate setting (12 μl sample per minute). A total of 30,000 events were acquired for each sample. Data analysis was performed using FCS Express version 3 Research Edition (*De Novo* Software, Los Angeles, CA, USA).

Epifluorescence microscopy

Bacterial cell suspensions, after ciprofloxacin exposure and recovery, were deposited on poly(D-lysine)-coated coverslips previously placed in the wells of a 48-well polystyrene plate. The plates were incubated at 37 °C for 45 min to allow the adhesion of *E. coli* cells onto the coverslips. The plates were then washed with PBS buffer and fixed for 10 min at room temperature with 4% paraformaldehyde solution. The plates were washed with PBS buffer and permeabilized for 5 min at room temperature with 1% Triton X-100 solution. The plates were washed with PBS buffer and a 7.5 μM DCR solution was added to the wells. The plates were then incubated at 37 °C for 30 min. The plates were washed with PBS buffer, and a 6 μg ml^{-1} fluorescein 5(6)-isothiocyanate (Sigma-Aldrich) solution was added to the wells in order to stain whole cells. The plates were then incubated at 37 °C for 30 min; afterwards, cells were washed twice, and the coverslips were mounted onto glass slides with DAKO (Dako, Glostrup, Denmark) mounting medium. These slides were then examined under an epifluorescence microscope (×100) in a Zeiss AX10 microscope using the Axio Vision Real 4.6 Software (Carl Zeiss MicroImaging GmbH, Jena, Germany).

RESULTS

DCR specificity

In order to verify DCR specificity for bacterial DNA, fixed cells were subjected to treatments with DNase, RNase and with both these enzymes before DCR labeling. The analysis of light-scatter parameters showed that there were no significant changes in particle size distribution after enzyme treatments (data not shown). DNase treatment (Figure 1b) yielded a very similar histogram to that obtained after the treatment with both enzymes (Figure 1c). Both of these histograms exhibited a slightly higher FL4 channel fluorescence comparing with the non-stained population (Figure 1a). RNase treatment yielded a very similar FL4 channel fluorescence distribution when compared with *E. coli* cells without treatment (Figures 1d and e). The fact that this stain is DNA-specific is further supported by the microscopic evaluation of DCR- and fluorescein 5(6)-isothiocyanate-stained *E. coli* cells, where DCR-stained *E. coli* chromosomes could be observed (Figure 1f).

Bacteriostatic versus bactericidal activity of ciprofloxacin

The ciprofloxacin MIC value obtained for *Escherichia coli* ATCC 25922 was 0.008 μg ml^{-1}. In order to assess if the concentrations used resulted in either a bacteriostatic or bactericidal effect, a time-kill study of bacterial cells exposed to ciprofloxacin concentrations ranging from 0.5 to 4 times the MIC value over 24 h incubation at 37 °C was performed (Figure 2). As it is known that bacterial growth phase can influence susceptibility profiles,[17] a pre-culture was performed to obtain a homogeneous population of cells in an active state as confirmed by propidium iodide staining (data not shown).

Time-kill curves showed that bacterial killing is concentration-dependent, for the rate of killing increases with progressively higher antibacterial concentrations. Incubation of *E. coli* cells with a ciprofloxacin concentration of 0.5× MIC did not result in a decreased bacterial cell count (Figure 2). For ciprofloxacin concentration of one time the MIC (0.008 μg ml^{-1}), the reduction in bacterial cell counts

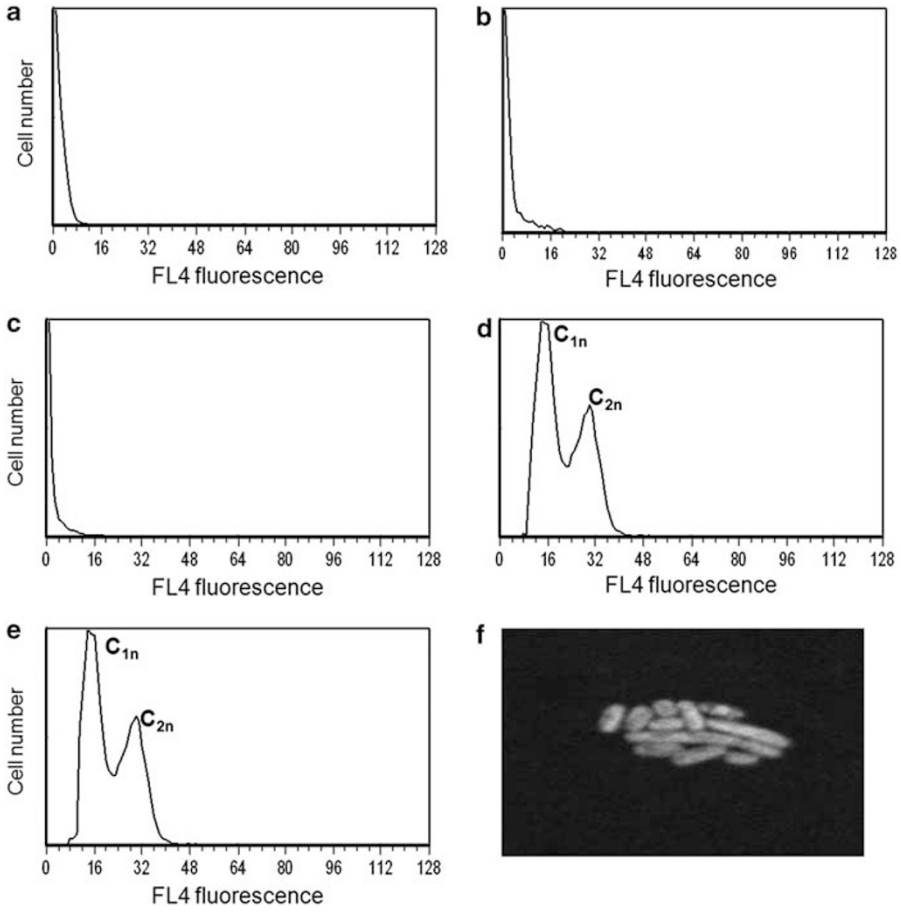

Figure 1 Effect of enzyme digestion on the DyeCycle Ruby (DCR) staining of fixed *E. coli* cells. (**a**) Untreated suspension without staining, (**b**) stained cell suspension after DNase treatment, (**c**) stained cell suspension after DNase and RNase treatment, (**d**) stained cell suspension after RNase treatment and (**e**) untreated and stained cell suspension; (**f**) epifluorescence microscopy images of *E. coli* cells immobilized on poly(D-lysine)-coated coverslips and incubated with DCR (red) and fluorescein 5(6)-isothiocyanate (green), as described in Materials and methods. A total of 20,000 events were collected for the flow cytometric analysis. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

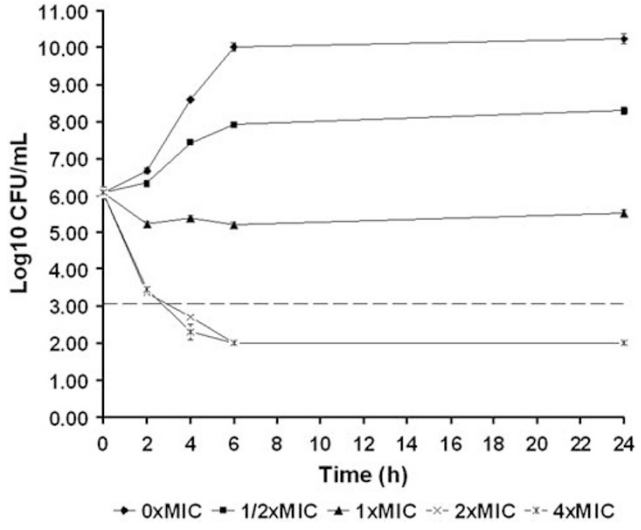

Figure 2 Viable counts for cultures of *E. coli*. Results are shown as the mean ± s.d. values for three experiments of control cultures (♦) and cultures treated at 37 °C during 24 h, with ciprofloxacin concentrations corresponding to 0.5 (■), 1 (▲), 2 (×) and 4 (*) times the MIC value determined.

was less than 3 $\log_{10}$ CFU ml^{-1}, indicative of a bacteriostatic effect. A bactericidal effect was obtained when *E. coli* cells were incubated with ciprofloxacin concentrations of two and four times the MIC (0.016 and 0.032 μg ml^{-1}, respectively) as can be seen by a reduction in bacterial cell counts of 4.0 $\log_{10}$ CFU ml^{-1} (Figure 2).

As bacteriostatic and bactericidal activities of ciprofloxacin were obtained for ciprofloxacin concentrations equaling one time and four times the MIC, respectively (Figure 2), those concentrations were chosen to perform the flow cytometric studies.

DNA histograms of *E. coli* cells not exposed to ciprofloxacin (Figure 3a) showed two peaks corresponding to one or two chromosome equivalents, and a ridge connecting the two peaks, which is indicative of actively growing cells. At 2 h of incubation, the majority of cells contained one chromosome equivalent; at 4 h, there is an increase of the peak corresponding to cells with two chromosome equivalents, which suffers a reduction at 6 h, where most cells contained one chromosome equivalent, as can be seen in the fluorescence microscopy image (Figure 4a). When analyzing DNA histograms of cells exposed to ciprofloxacin at one time the MIC (bacteriostatic effect) (Figure 3b), there is a decrease in peak resolution throughout incubation time and all DNA histograms are very similar, with cells containing one or two chromosome equivalents. These cells

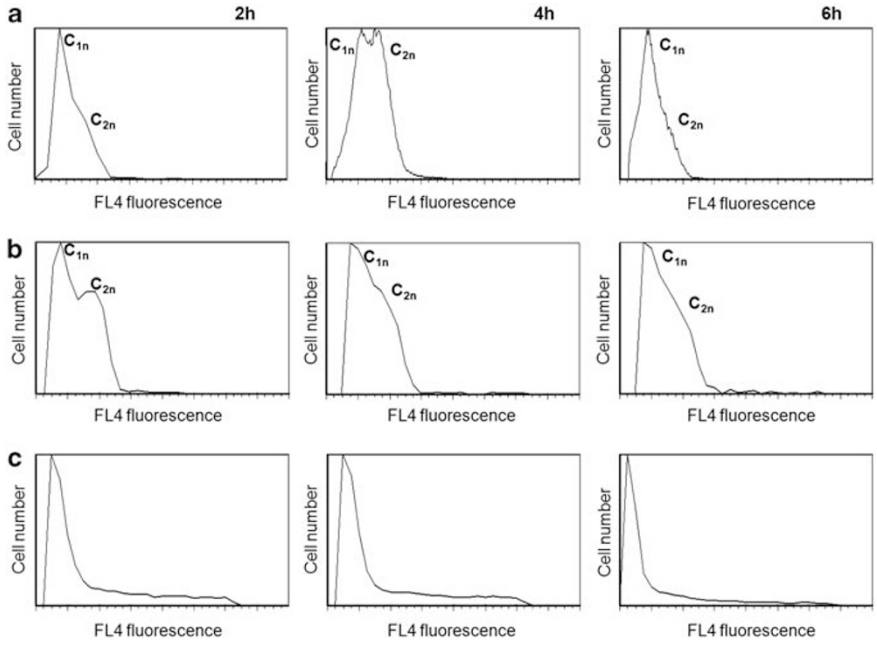

Figure 3 Effect of ciprofloxacin concentration on *E. coli* DNA content. Histograms show the distribution of DyeCycle Ruby fluorescence intensity (FL4 channel) in a linear scale using *E. coli* cultures (**a**) not treated or exposed to a ciprofloxacin concentration of: (**b**) one time the MIC and (**c**) four times the MIC. A total of 30,000 events were collected for this analysis.

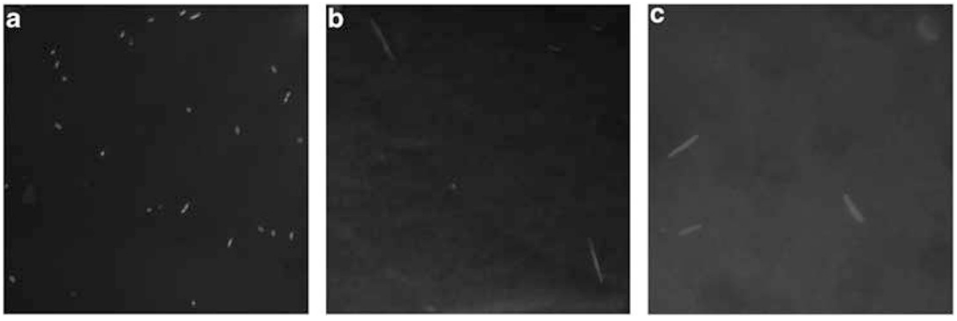

Figure 4 Epifluorescence microscopy images of *E. coli* cells after 6 h of incubation. (**a**) Control, (**b**) cells exposed to a ciprofloxacin concentration of one time the MIC and (**c**) four times the MIC. Cells were immobilized on poly(D-lysine)-coated coverslips and incubated with DyeCycle Ruby (red) and fluorescein 5(6)-isothiocyanate (green), as described in Materials and methods. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

exhibited a high filamentous structure where the chromosomes can be observed (Figure 4b). Cells exposed to a ciprofloxacin concentration of four times the MIC (bactericidal effect) (Figure 3c) exhibited DNA histograms with only one peak, with lower fluorescence intensities and a broadening of the histogram for higher fluorescence intensities. Because of this fact, the number of chromosome equivalents cannot be accurately determined. These findings are further supported by the microscopic visualization of the cells (Figure 4c) in which a filamentous structure can be observed, and because of the low fluorescence intensities the chromosomes cannot be fully distinguished.

DISCUSSION

As fluorescent stains with the Vybrant DyeCycle series have proven to be effective in staining live eukaryotic cells[18] and fixing prokaryotic cells, such as those of the Gram-negative bacterium *Caulobacter crescentus*,[19] the present work investigates the ability of DCR to stain bacterial DNA and further evaluates ciprofloxacin activity in the Gram-negative bacterium *E. coli*.

As one of the major characteristics of these fluorescent stains is that they bound selectively to eukaryotic DNA, it was important to clarify if DCR was also specific for bacterial DNA. DCR proved to be specific for bacterial DNA, as DNase treatments yielded a fluorescence distribution similar to that without DCR labeling, while RNase treatment yielded a fluorescence distribution similar to that with DCR labeling.

The dose-dependent bactericidal activity of ciprofloxacin is in accordance with previous studies.[20] The marked decrease in bacterial viability could be explained by the fact that, in Gram-negative bacteria, ciprofloxacin is directed at DNA gyrase, which results in rapid blockage of DNA synthesis.[11] Despite this decrease in bacterial viability, flow cytometric cell counts were not affected (data not shown), which is in agreement with the results obtained by other authors.[21]

As ciprofloxacin exhibited bacteriostatic and bactericidal activities at the concentrations tested, the next step was to develop a flow cytometric method that allowed the assessment of ciprofloxacin activity.

Previous studies reported that ciprofloxacin does not have a direct action on bacterial cell membrane integrity even at high concentrations.[21,22] Therefore, propidium iodide was not an effective indicator of ciprofloxacin susceptibility, and was not considered for this work.

Another approach described in the literature to evaluate ciprofloxacin exposure consisted of the evaluation of membrane potential[21,22] using bis-oxonol. As ciprofloxacin causes membrane depolarization at high concentrations, this could be seen as a good method to evaluate ciprofloxacin susceptibility. However, membrane potential is reversible, and other studies have shown that depolarized cells are able to grow when placed in fresh medium;[23] thus, the percentage of stained cells will not reflect bacterial viability.

It is known that ciprofloxacin has a direct action on chromosomal DNA replication and fragmentation;[24] hence, we intended to evaluate its activity by flow cytometric analysis of intracellular of DNA content using DCR.

The results obtained clearly showed two distinct DNA patterns related to two distinct ciprofloxacin activities: bacteriostatic and bactericidal. Ciprofloxacin bacteriostatic activity is due to an inhibition of DNA synthesis and cell growth.[25] *E. coli* DNA histograms corresponding to ciprofloxacin bacteriostatic activity showed a similar profile and fluorescence intensities over the 6 h of incubation time. All DNA histograms showed two peaks corresponding to one and two chromosome equivalents, as a result the fluorescence intensity values obtained. The resolution of the histograms decreased with time, indicating that rifampicin and cefalexin treatments are no longer effective, perhaps because of ciprofloxacin action in bacterial DNA. These cells exhibited a highly filamentous morphology, which is in agreement with previous studies.[21] In these filamentous cells, one or two chromosomes can be observed by fluorescence microscopy, further supporting the results obtained by flow cytometry.

Ciprofloxacin bactericidal activity is thought to be related to the release of free DNA ends from the DNA gyrase–quinolone complexes,[25] leading to chromosomal DNA fragmentation.[24] *E. coli* DNA histograms corresponding to ciprofloxacin bactericidal concentration showed similar fluorescence intensities throughout the 6 h of incubation. Because of the high ciprofloxacin concentrations used, cellular repair mechanisms were not able to increase DNA synthesis[26] and, therefore, a complete inhibition of DNA synthesis is observed. Furthermore, the lower fluorescence intensities obtained and higher fluorescence broadening without visible peaks could be related to DNA fragmentation,[27] because DNA fragments have a compromised ability to be stained with intercalating dyes,[24] such as DCR, resulting in lower relative DCR fluorescence. These results were further supported by microscopic images, which showed a low staining level and the inability of visualizing chromosomes. These cells have a filamentous morphology with larger cell size than that of the control culture, but with smaller cell size when compared with cells incubated with one time the MIC (bacteriostatic effect). This cell filamentation as a result of ciprofloxacin exposure is well documented[21,28] and can be seen as an indicative of the induction of SOS response.[29]

Because of the characteristics of the DNA-selective fluorescent stain used, DCR, it was possible to verify the applicability of this fluorescent stain to analyze bacterial DNA patterns for the purpose of studying ciprofloxacin activity. This flow cytometric analysis can also be applied to other antibiotics that are known to have different effects on bacterial DNA, such as aminoglycosides.

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