Ethidium Monoazide for DNA-Based Differentiation of Viable and Dead Bacteria by 5′-Nuclease PCR

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ABSTRACT

PCR techniques have significantly improved the detection and identification of bacterial pathogens. Even so, the lack of differentiation between DNA from viable and dead cells is one of the major challenges for diagnostic DNA-based methods. Certain nucleic acid-binding dyes can selectively enter dead bacteria and subsequently be covalently linked to DNA. Ethidium monoazide (EMA) is a DNA intercalating dye that enters bacteria with damaged membranes. This dye can be covalently linked to DNA by photoactivation. Our goal was to utilize the irreversible binding of photoactivated EMA to DNA to inhibit the PCR of DNA from dead bacteria. Quantitative 5′-nuclease PCR assays were used to measure the effect of EMA. The conclusion from the experiments was that EMA covalently bound to DNA inhibited the 5′-nuclease PCR. The maximum inhibition of PCR on pure DNA cross-linked with EMA gave a signal reduction of approximately -4.5 log units relative to untreated DNA. The viable/dead differentiation with the EMA method was evaluated through comparison with BacLight™ *staining (microscopic examination) and plate counts. The EMA and* BacLight *methods gave corresponding results for all bacteria and conditions tested. Furthermore, we obtained a high correlation between plate counts and the EMA results for bacteria killed with ethanol, benzalkonium chloride (disinfectant), or exposure to 70°C. However, for bacteria exposed to 100°C, the number of viable cells recovered by plating was lower than the detection limit with the EMA method. In conclusion, the EMA method is promising for DNA-based differentiation between viable and dead bacteria.*

INTRODUCTION

DNA-based methods such as PCR have been increasingly used for the rapid, sensitive, and specific detection of pathogens (1). However, there are still limitations in the usage of nucleic acid-based diagnostics. A major obstacle is the lack of differentiation between DNA from viable and dead microorganisms (2–4). The DNA molecule may remain intact even though the organism is dead (5–9). RNA has been used as a viable/dead marker due to its intrinsic instability (3,4,10,11). However, accurate viable/dead measurements are difficult using RNA as a target. The gene analyzed has to be continuously expressed, and the transcript has to be relatively unstable.

Most disinfection and preservation techniques are aimed at either inactivating or removing potential pathogens. Generally, the bacteria die before the DNA is destroyed. The ability of the nucleic acids from dead cells to generate PCR signals is affected by the preservation technique, disinfection treatment, and organism (2,12). It is therefore a great demand for sample preparation methods related to whether the organisms are viable or dead to fully exploit the potential of PCR in microbiological diagnostics. An aspect that has not yet been used in PCR analyses is the physical differences between viable and dead cells. This concept, however, is widely used in both microscopy and flow cytometry. Ethidium monoazide (EMA) (Molecular Probes Europe BV, Leiden, The Netherlands) has been used as a live-dead stain in several of these assays (13–19). EMA is a DNA intercalating agent (20), and photolysis of EMA with visible light produces a nitrene (21) that forms stable monoadducts when bound to DNA (22,23). The free EMA in solution is photolyzed simultaneously and converted to hydroxylamine (21) and is no longer capable of covalent attachment (24).

Our goal in the current study was to utilize the irreversible binding of photoactivated EMA to DNA to inhibit the PCR of DNA from dead bacteria. Quantitative 5′-nuclease PCR was used to measure the effect of EMA. The maximum inhibition of PCR on pure DNA cross-linked with EMA gave a signal reduction (EMASR) of approximately -4.5 log units relative to untreated DNA. *E. coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* were

tested as model organisms. Heat, disinfectants, and alcohol were used as killing agents. There was a high correlation between plate counts and the EMASR for the bacteria and conditions tested. The novelty of the approach is the use of an agent (EMA) that selectively modifies the DNA in dead cells so that only the DNA in viable cells can be PCR amplified.

MATERIALS AND METHODS

Bacterial Strains, Media, and Cultures

Model organisms used were *E. coli* O157:H7 NCTC 1200 (National Collection of Type Cultures, Colindale, London), *E. coli* O157:H7 MATFORSK (MF) strain 667, *E. coli* O157:H7 isolate 604 (Y. Wasteson, Veterinary Institute, Oslo, Norway), *L. monocytogenes* L028 wild-type (Institut Pasteur, Paris, France), *L. monocytogenes* EGDe serotype 1/2a (Institut Pasteur), *L. monocytogenes* MATFORSK strain 54 (L.-M. Rørvik, Norwegian College of Veterinary Medicine, Oslo, Norway), *L. monocytogenes* DSMZ 20600 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) type strain, *Salmonella* sp. ATCC 13311 (ATCC, Rockville, MD, USA), and *Salmonella* sp. MF strain 16.

Listeria was grown in Brain Heart Infusion (BHI) media at 30°C. *E. coli* and *Salmonella* were grown in BHI media at 37°C. The cultures were serially diluted in peptone water. The cfu were determined by plating 0.1 mL of each dilution onto BHI agar and incubating at 30°C (*Listeria*) or 37°C for 1–2 days. All agars and media were from Oxoid Ltd. (Basingstoke, Hampshire, UK).

Viable/Dead Staining for Microscopy

The two-color fluorescence assay LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes Europe BV) was used to stain the organisms for microscopy. SYTO® 9 stain (Molecular Probes Europe BV) generally labels all bacteria in a population green, while propidium iodide penetrates only bacteria with damaged membranes and labels these red (i.e., reducing the SYTO 9 stain fluorescence when both dyes are present). To compare *Bac*Light staining and staining with EMA in combination with 4-6-di-amidino-2-phenyl-indol (DAPI) (stains nucleic acids), 1 mL of the respective samples was pelleted at 10 000× *g* for 7 min. The samples were stained with *Bac*Light, following the manufacturer's instructions, incubated for 15 min, and filtered through Osmonic Polycarbonate Filters 25 mm (Osmonic, Minnetonka, MN, USA), washed with peptone water, and mounted on slides.

The samples were also stained with 1 µg/mL EMA for 5 min on ice in the dark, subjected to a 650-W halogen lamp for 1 min, and filtered as described earlier. The EMA-stained filters were subsequently counterstained with 10 µg/mL DAPI.

Heat and Disinfection Treatments

Overnight stationary phase cultures were used in the experiments. The number of bacteria was determined by plate counting, and samples from the same culture were used in each parallel experiment (i.e., the amount of cfu was equal in each experimental series). The cultures were either heat treated for 5 min (72°C or 100°C) or pelleted at 5000–6000× *g* for 7 min at 4°C and resuspended in the killing agents (96% ethanol, 70% isopropanol, or 500 ppm benzalkonium chloride) and incubated at 20°C for 5–30 min. Then the samples were pelleted and resuspended in equal volumes of BHI media as described earlier.

EMA Cross-Linking

EMA bromide (phenanthridium, 3-amino-8-azido-5-ethyl-6-phenyl bromide) was purchased from Molecular Probes Europe BV. Five micrograms of solid were dissolved in 0.5 mL water in dark microcentrifuge tubes and further diluted. The solutions were stored at -20°C and kept on ice when used. EMA is potentially carcinogenic and should be treated according to the manufacturer's safety instructions. The light source was an OSRAM SLG 1000 (Osram AS, Drammen, Norway) with a 650-W halogen light bulb, which was placed 20 cm from the sample tubes. The microcentrifuge tubes were placed on ice to minimize elevated temperature in the samples.

DNA Isolations

DNA was isolated using PrepMan™ Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. The samples (0.10–0.15 mL) were added to 0.2 mL PrepMan extraction reagent and incubated at 56°C for 30 min. The samples were then vortex mixed for 10 s, boiled for 8 min, and centrifuged at 16 000× *g* for 5 min. The supernatants were diluted and subjected to 5′-nuclease PCR.

5′-Nuclease PCR Assay

Primers and probes for *E. coli* O157:H7 were constructed for the attaching and effacing gene intimin (*eae*). The assay constituted forward primer 5′-CTGAATTTGATACCTTAAGTGCAGC-3′, reverse primer 5′-AGGCACGCCTAAACCTATAGCT-3′, and probe 5′-TCTCCTTGCTCATCTTTAGGATAAATTCTTTCACA-3′. Primers and probes for the detection and quantification of *L. monocytogenes* have been previously described (25). *Salmonella* was quantified using the TaqMan® *Salmonella* PCR Amplification/Detection Kit (Applied Biosystems) (26).

The 5′-nuclease PCR on DNA from *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* was carried out as previously described (25,27). PCR samples and controls were prepared in triplicate. PCR products were detected directly by monitoring the increase in fluorescence from the dye-labeled-specific DNA probes. The reporter dye, carboxyfluorescein (FAM) was covalently linked to the 5′ end of the oligonucleotides. The fluorescence of the reporter dye was then quenched by 6-carboxy-N, N, N′, N′-tetramethyl-rhodamine (TAMRA) located at the 3′ ends. The reporter signal was normalized to the emission of an internal reference dye (ROX-6-carboxy-X-rhodamine). The fluorescence signal was

Table 1. Effect of Light Exposure and EMA Concentration on DNA Inactivation

	Light Exposure[a]					
EMA (μg/mL)	5 s	15 s	30 s	45 s	1 min	3 min
10	-3.13 ± 0.18	-3.38 ± 0.20	-3.73 ± 0.12	-3.85 ± 0.13	-3.39 ± 0.18	-2.64 ± 0.13
100	-2.06 ± 0.08	-3.60 ± 0.34	-4.61 ± 0.06	-4.16 ± 0.30	-3.85 ± 0.45	-4.61 ± 0.06

[a]EMA was cross-linked to DNA for the given time periods. Fractions (log_{10}) of the treated DNA giving PCR signals relative to untreated DNA are shown. Standard deviations from three replicates are included.

Table 2. Effect of Photolysis of EMA before Cross-Linking to DNA

	EMA Photolysis[a]					
EMA (μg/mL)	5 s	15 s	30 s	45 s	1 min	3 min
10	-1.36 ± 0.27	-0.29 ± 0.42	-0.17 ± 0.26	-0.20 ± 0.47	-0.02 ± 0.15	-0.04 ± 0.07
100	-4.23 ± 0.31	-0.89 ± 0.04	-0.53 ± 0.05	-0.67 ± 0.12	-0.61 ± 0.09	-1.24 ± 0.02

[a]EMA was photolysed for the given time periods before cross-linking to DNA. Fractions (log_{10}) of the treated DNA giving PCR signals relative to untreated DNA are shown. Standard deviations from three replicates are included.

plotted as ΔR_n, which was the normalized reporter signal minus background, against the number of cycles. The threshold cycles (C_T) was determined for each amplification plot by setting a fixed threshold (ΔR_n 0.03) above the baseline (background). Different amplifications could then be compared by their respective C_Ts since calculated threshold values are proportional to the number of target copies present in the sample (28). The C_T values were plotted against log input cfu, and the corresponding standard curves were used to estimate slopes and square regression coefficients (R^2) by linear regression to evaluate the quantitative properties of the assays (28,29). The efficiencies (E) were calculated using the equation $E = 10^{-1/s} - 1$, where s is the slope of the regression curve (30). C_T values for the non-EMA-treated samples were subtracted from the C_T values for the corresponding EMA-treated samples. This gave ΔC_T values for the effect of the EMA treatments, independent of the amount of template added. Finally, the ΔC_Ts were divided by the slope of the regression curve to calculate the log_{10} of the EMASR. EMASR represents an approximation of the DNA fraction in the EMA-treated samples that than be PCR amplified.

Correlation Analyses

The Pearson correlation coefficient was used to measure the degree of the linear relationship between the plate counts and the 5-nuclease PCR data. The correlation coefficient was calculated with the following formula for the two variables x and y; $r = \Sigma (x - m_x)(y - m_y)/(n - 1)s_x s_y$, where m_x and s_x are the sample mean and the standard deviation for the first variable, and m_y and s_y are the sample mean and standard deviation for the second variable.

RESULTS

Evaluation of the Quantification Assays

The reproducibility of the PrepMan Sample Preparation Reagent and the 5′-nuclease primer and probe systems were tested using *E. coli* O157:H7 NCTC 1200, *L. monocytogenes* EGDe, and *Salmonella* sp ATCC 13311. Dilution series were made from both cells before DNA purification and from pure DNA. Slopes of the standard curves and their corresponding amplification efficiencies, together with the square regression coefficients (R^2), were then determined. The slopes of the regression curves in all cases were between -3.3 and -3.4. All three detection systems gave amplification efficiencies in the range from 0.98 to 1.0 and an R^2 from 0.995 to 0.999. These values were subsequently used in quantifying the effect of the EMA treatment.

Influence of the EMA Fluorescence on the 5′-Nuclease PCR Assay

The influence of EMA fluorescence on the 5′-nuclease PCR assay was examined. The raw spectra and the multicomponent spectra were determined. The spectra were not affected by EMA content below 200 μg/mL. Higher contents gave a proportional decrease in the fluorescence intensity of the background reporter signal (FAM) (data not shown).

Optimization and Evaluation of the EMA/DNA Cross-Linking Process

EMA was cross-linked to pure DNA using different irradiation times and EMA concentrations. The highest PCR inhibition was obtained using DNA cross-linked with 100 μg/mL EMA and irradiation for more than 30 s. This resulted in an EMASR in the range from -3.9 to -4.6 logs (Table 1).

To control the efficiency of photoly-

Table 3. Effect of EMA Concentration and Exposure Time for Differentiation between Viable and Isopropanol-Killed *E. coli* O157:H7

EMA (µg/mL)	EMA Exposure[a] 5 s	30 s	1 min	5 min	10 min
Viable					
10	-0 ± 0.11	-0.20 ± 0.05	-0.35 ± 0.04	-0.39 ± 0.03	-0.31 ± 0.06
100	-0.20 ± 0.05	-0.26 ± 0.06	-0.37 ± 0.04	-0.24 ± 0.03	-0.25 ± 0.03
Dead					
10	-1.25 ± 0.06	-1.22 ± 0.03	-1.30 ± 0.06	-1.12 ± 0.04	-1.44 ± 0.08
100	-1.70 ± 0.03	-1.62 ± 0.07	-2.03 ± 0.07	-2.31 ± 0.07	-2.44 ± 0.18

[a]The cells were exposed to EMA for the given time periods in the dark before cross-linking with light for 1 min. Fractions (log_{10}) of the treated DNA giving PCR signals relative to untreated DNA are shown. Standard deviations from three replicates are included.

sis (i.e., the inactivation of free EMA in solution), EMA was irradiated without DNA, as described earlier. The maximum effect was obtained for irradiation between 30 s and 1 min (Table 2). The EMASRs were approximately -0.5 logs for 100 µg/mL EMA and -0.2 logs for 10 µg/mL EMA. A high concentration (333 µg/mL) of photolysed EMA inhibited the PCR regardless of irradiation time (data not shown).

Finally, we tested whether DNA cross-linked with EMA had an effect on the detection of native DNA. This was done by subjecting native DNA, DNA cross-linked with EMA, and a mixture of the two to 5′-nuclease PCR. There was no influence from the EMA-cross-linked DNA on the detection of native DNA (data not shown).

Evaluation of the EMA Assay Using Isopropanol-Killed Cells

The bacteria were either untreated or killed with 70% isopropanol (see Materials and Methods). *E. coli* O157:H7 NTNC 1200 was used as a model to optimize the exposure time and concentration of EMA (Table 3). There was no detectable difference in the PCR signals from viable *E. coli* O157:H7 with 10 or 100 µg/mL EMA added for incubation up to 10 min. Incubation with 100 µg/mL EMA for 5 min or more gave the best inactivation with a EMASR of approximately -2.5 logs for the killed cells.

A set of three *E. coli* O157:H7 strains, two *Salmonella* strains, and three *L. monocytogenes* strains were tested using a standardized protocol with 100 µg/mL EMA, incubation in the dark for 5 min, with subsequent irradiation for 1 min. The bacteria were killed as described earlier. All the strains tested gave a good differentiation between the viable and dead bacteria (Figure 1). However, there was, a relatively high amount of dead bacteria in some of the overnight cultures, resulting in inhibition for the corresponding viable controls (determined by *Bac*Light staining).

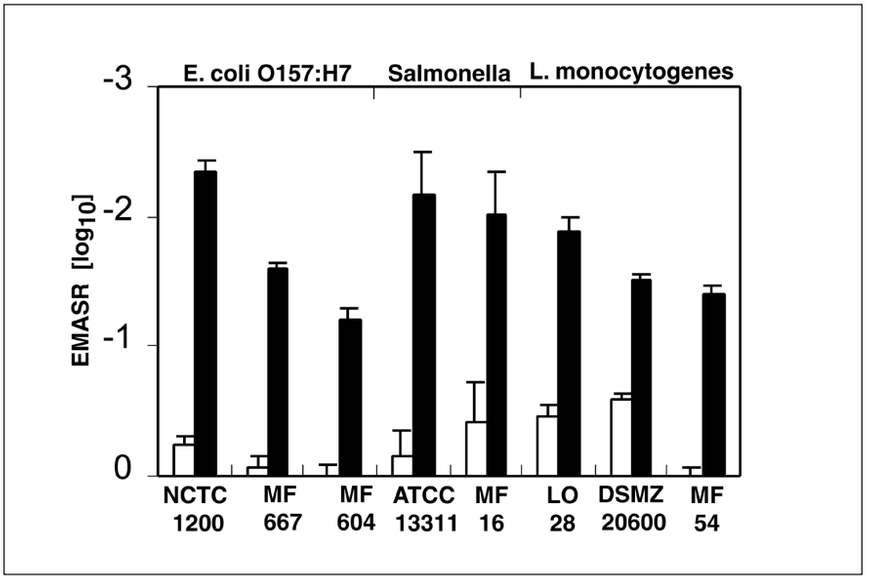

Figure 1. Evaluation of the EMA assay on viable (white bars) or isopropanol-killed bacteria (black bars). The EMASR is shown (see Materials and Methods for definition). The cells were exposed to 70% isopropanol for 30 min. The error bars are the standard deviations of three replicates.

Comparison of the EMA Assay and Plate Counts Using Heat- or Disinfectant-Treated Cells

We tested heat, ethanol, or benzalkonium chloride as disinfecting agents. The *Salmonella* strain was relatively resistant to the conditions tested, while the *L. monocytogenes* strain was sensitive to heat and benzalkonium chloride (Figure 2). The correlation between the log cfu and the EMASR for the conditions tested was determined (see Materials and Methods). A Pearson correlation of 0.919 for all conditions (except 100°C) indicated a high linear relation between log cfu and the DNA signal. The more than -7 log reduction in cfu for bacteria killed at 100°C was probably below the detection limit for the EMA assay. The maximum differentiation between the EMA-stained DNA and DNA without EMA is an EMASR of approximately -4.5 log (see Table 1).

Comparison of EMA and *Bac*Light Staining Using Microscopic Examination

There was a good correspondence between EMA staining and the *Bac*-

Light system, using microscopic examinations for the bacteria and killing agents tested here (empirical data). Furthermore, there was a clear difference at the cell level between bacteria stained with EMA (potentially dead) and the bacteria that did not stain (potentially viable). Figure 3 illustrates the correspondence between EMA and *Bac*Light staining for *Salmonella typhimurium*. There are dead cells present in the "viable" population of *S. typhimurium*, which was stained with EMA (Figure 3C). This coincides with results of staining with *Bac*Light (Figure 3E) (live cells give green fluorescence, and dead cells give red/brown fluorescence). Nearly all the killed cells were stained with EMA (Figure 3D). These cells were also stained as dead using *Bac*Light (Figure 3F). The reason for presenting qualitative *Bac*Light data is that we found it difficult to extract quantitative information from microscope examinations.

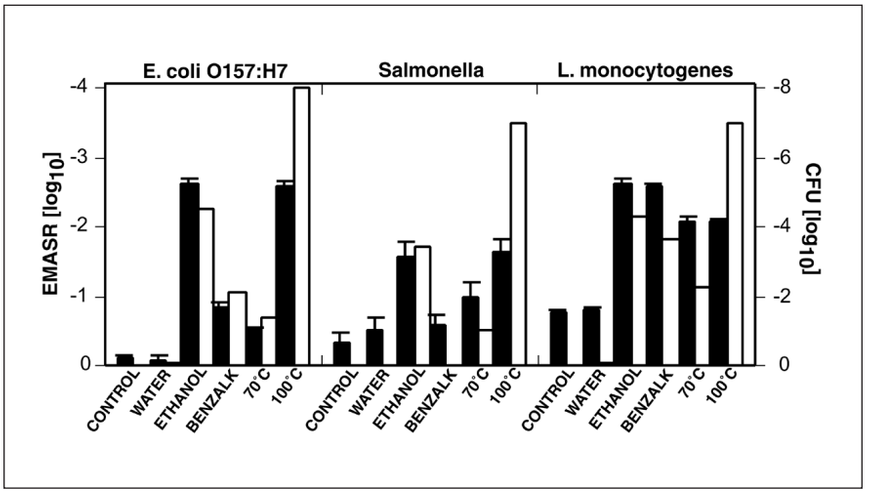

Figure 2. Comparison of EMA assay (black bars) and cfu (white bars) for bacteria treated for 5 min under different conditions. The EMASR is shown (see Materials and Methods for definition). The error bars are the standard deviations of three replicates. Values are shown for the cfu counts after treatment relative to the counts before treatment. For 100°C, no viable cells were recovered in a 100-µL volume for *Salmonella* or *L. monocytogenes*. The bars represent the detection limits. The detection limit represents that less than one bacterium can regain the growth of the bacteria in the 100 µL that were tested. The strains used are *E. coli* MF 667, *Salmonella* sp. ATCC 13311, and *L. monocytogenes* DSMZ 20600. BENZALK, benzalkonium chloride.

DISCUSSION

EMA Cross-Linking on Pure DNA

The photolysis of EMA was shown to be a first order reaction with respect to time (31), and the half-life was calculated as $\tau = 2.71 \times 10^2$ s when daylight light bulbs were used (32). Cantrell et al. (33) found significant differences in the rates of photolysis that resulted from variations in light intensity. However, the final extent of adduct formation on prolonged irradiation was identical. Our experience is that a maximum inhibition of the PCR signal from -3.9 to -4.6 logs was obtained after 15–30 s of irradiation.

Unbound EMA molecules at the time of photoactivation will react with solvent and be "inactivated." These molecules are no longer capable of covalent attachment (32,34,35). With our light source, maximum inactivation was obtained between 30 s and 1 min. A light exposure period of 1 min was therefore chosen for our standardized assay. The reason was both to ensure maximum cross-linking to DNA and to inactivate the free EMA (so it will no longer react with DNA after lysis of the bacteria).

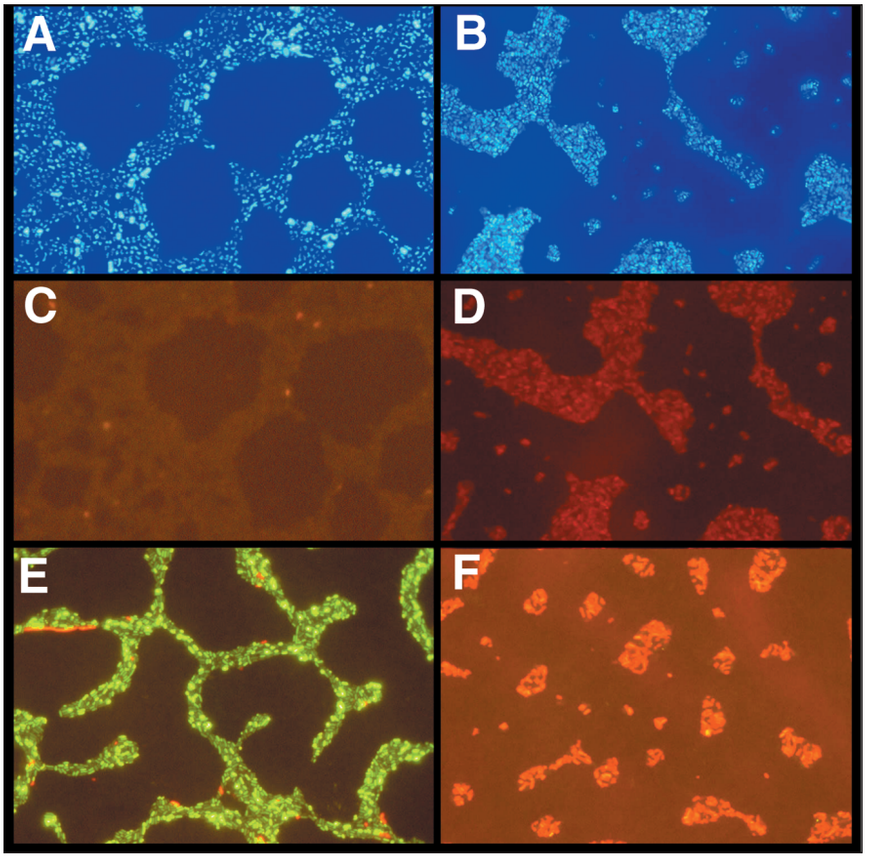

Figure 3. Epifluorescence microscopy of cells of *S. typhimurium*. Live cells (overnight culture suspended in peptone water) stained with DAPI (A), EMA (C), and *Bac*Light (E). Killed cells (treated with isopropanol) stained with DAPI (B), EMA (D), and *Bac*Light (F).

Optimization of the EMA Viable/Dead Assay on Bacteria

A study by Yielding et al. (36) suggested that EMA and *Salmonella* spp. cells reach equilibrium after 3–4 min. This is in accordance with our observations that maximum differentiation between viable and dead bacteria was obtained after a 5-min exposure to EMA in the dark before cross-linking. One hundred micrograms per milliliter gave a better discrimination between viable and dead bacteria than 10 µg/mL EMA (see Table 3). We standardized our viable/dead assay using an EMA concentration of 100 µg/mL, with incubation in the dark for 5 min, and light exposure as described earlier. It is important with an EMA exposure that is sufficient to enable EMA entry into dead cells while the viable cells remain unstained since the viable/dead measurements are based on the EMASR between stained and unstained DNA.

Generally, there were slightly lower PCR signals for the viable controls treated with EMA than for the viable cells with no EMA added. The cells were from overnight cultures that had reached the stationary phase. DNA from dead cells at the time of sample collection would also be stained with EMA, contributing to the lower PCR signal from the EMA-treated viable controls than from the non-EMA-treated controls. The *Bac*Light results confirmed the presence of dead cells in the overnight cultures with reduced signal after EMA treatment.

Evaluation of the EMA Viable/Dead Assay

Four physiological states are identified in the range from viable to dead bacteria. These states are reproductively viable, metabolically active, intact, and permeabilized dead cells (reviewed in 15). The permeation of bacteria by dyes is complex because of the structure of the bacterial cell wall and because the marked differences in the efflux pumps in the different species of bacteria (37). In the Gram-negative bacterial wall, the complex structure of the outer membrane represents the major permeability barrier, while the corresponding barrier in the Gram-positive bacteria is the peptidoglycan layer.

All viable/dead measurements have certain inherent limitations or artifacts. As described earlier, viable/dead dyes may not strictly correlate with cell viability. On the other hand, viable/dead measurements based on cell growth require that the bacteria regain growth under the conditions tested. Viable but starved or stressed bacteria may not be recovered, resulting in an underestimation in the presence of the viable bacteria (24,38). The number of viable cells estimated by plating may also be biased due to aggregation of the bacteria (39).

We tested conditions in our study that did not kill all the bacteria present in the samples. Sub-lethally damaged cells should thus be expected. There was a good linear correlation between the log cfu and the log of the DNA signals obtained with the EMA method for the controls and bacteria exposed to water, ethanol, benzalkonium chloride, or heat at 70°C (see Figure 2). However, the two subpopulations of cells (cells that stain with EMA, and cells that can recover growth) are not necessarily the same. The correlation could be that the log differences between these populations are linear for the conditions tested. The recoverable cells probably underestimate the number of viable cells since the sub-lethally injured cells may not regain growth. For the boiled samples, the reduction in cfu was lower than the EMASR. This could be due to the presence of viable bacteria that cannot be recovered on the media used, the number of viable cells being below the detection limit for the EMA method, or both.

Potential for Analyses of Mixed Populations

None of the current viable/dead staining-based measurements are suited for analyzing different bacteria in mixed populations (13). This is a serious limitation with these methods. However, with the EMA method, we should be able to analyze mixed populations. All the bacteria in the populations are stained as either viable or dead. It should be possible to individually measure the EMASRs for the different bacteria in the population with PCR. Ultimately, the EMA method may help us to better understand the interaction among the approximately 99% of the bacteria in the environment that we are unable to grow in culture (40).

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