

# Comparative Analysis and Limitations of Ethidium Monoazide and Propidium Monoazide Treatments for the Differentiation of Viable and Nonviable *Campylobacter* Cells

Diana Seinige, Carsten Krischek, Günter Klein, Corinna Kehrenberg

Institute for Food Quality and Food Safety, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany

The lack of differentiation between viable and nonviable bacterial cells limits the implementation of PCR-based methods for routine diagnostic approaches. Recently, the combination of a quantitative real-time PCR (qPCR) and ethidium monoazide (EMA) or propidium monoazide (PMA) pretreatment has been described to circumvent this disadvantage. In regard to the suitability of this approach for *Campylobacter* spp., conflicting results have been reported. Thus, we compared the suitabilities of EMA and PMA in various concentrations for a *Campylobacter* viability qPCR method. The presence of either intercalating dye, EMA or PMA, leads to concentration-dependent shifts toward higher threshold cycle ( $C_T$ ) values, especially after EMA treatment. However, regression analysis resulted in high correlation coefficient ( $R^2$ ) values of 0.99 (EMA) and 0.98 (PMA) between *Campylobacter* counts determined by qPCR and culture-based enumeration. EMA (10  $\mu\text{g/ml}$ ) and PMA (51.10  $\mu\text{g/ml}$ ) removed DNA selectively from nonviable cells in mixed samples at viable/nonviable ratios of up to 1:1,000. The optimized EMA protocol was successfully applied to 16 *Campylobacter jejuni* and *Campylobacter coli* field isolates from poultry and indicated the applicability for field isolates as well. EMA-qPCR and culture-based enumeration of *Campylobacter* spiked chicken leg quarters resulted in comparable bacterial cell counts. The correlation coefficient between the two analytical methods was 0.95. Nevertheless, larger amounts of nonviable cells ( $>10^4$ ) resulted in an incomplete qPCR signal reduction, representing a serious methodological limitation, but double staining with EMA considerably improved the signal inhibition. Hence, the proposed *Campylobacter* viability EMA-qPCR provides a promising rapid method for diagnostic applications, but further research is needed to circumvent the limitation.

*Campylobacter* spp. are the most frequently reported cause of food-borne disease in the European Union and a major concern for public health (1). In 2010, more than 213,200 cases were reported in the European Union, and the Robert Koch Institute reported 48.56 confirmed cases per 100,000 inhabitants in Germany in 2011 (1, 2). Contaminated poultry meat and cross-contaminated food are known to be the major sources of infection and have been identified as significant risk factors (1–3). As the cultivation of *Campylobacter* spp. is time-consuming and unsuitable for detecting viable but nonculturable (VBNC) cells, PCR-based methods have been developed for the detection and quantification of thermophilic *Campylobacter* in food, feed, or fecal or water samples (4–8). However, the lack of differentiation between viable and nonviable cells (due to the persistence of DNA after cell death) is a crucial point that limits the implementation of these approaches for routine diagnostic applications, even if a rapid quantification method is urgently needed (9). To circumvent this disadvantage, intercalating dyes like ethidium monoazide (EMA) were applied to bacterial samples before PCR analysis, allowing a live/dead discrimination of some bacterial species (10). These dyes enter bacteria with damaged cell membranes and covalently bind to genomic DNA after photoactivation. As a consequence, the PCR amplification of DNA from dye-treated nonviable cells is inhibited and DNA is cleft or lost during the extraction process (10, 11). While satisfactory EMA-PCR results have been reported, e.g., for *Escherichia coli* and *Salmonella enterica*, inconsistent findings were obtained for *Campylobacter jejuni*, possibly depending on the strains or conditions tested (9, 11, 12). Since EMA is known to penetrate viable cells to some extent, the chemical substance propidium monoazide (PMA), which is more highly charged, was

subsequently tested for its ability to differentiate between viable and nonviable cells. Results for various bacterial species, e.g., *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica* serovar Typhimurium, or *Serratia marcescens*, indicated an advantage of PMA over EMA, likely due to its reduced ability to penetrate live cells (13, 14). However, studies reporting the pretreatment of *Campylobacter* samples with intercalating dyes are rare (9, 15, 16) and do not include a direct comparison of EMA and PMA applications.

Therefore, the aim of the present study was to comparatively analyze the applicability of EMA and PMA at various concentrations to discriminate between viable and dead *Campylobacter* cells by quantitative real-time PCR (qPCR).

## MATERIALS AND METHODS

**Bacterial strains and species confirmation.** Reference strain *C. jejuni* DSM 4688 (Leibniz-Institut, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was used for the validation experiments. In addition, *Campylobacter coli* DSM 4689 (Leibniz-Institut) and 16 avian *Campylobacter* field isolates (7 of *C. jejuni* and 9 of *C. coli*), representing a part of the strain collection of the Institute for Food

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Address correspondence to Corinna Kehrenberg, corinna.kehrenberg@tiho-hannover.de.

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Quality and Food Safety, were recovered from  $-80^{\circ}\text{C}$  storage and included in the study. Strains were cultivated for 24 h (or 48 h for a culture-based enumeration) on modified charcoal cefoperazone desoxycholate agar (CCDA) plates (Oxoid, Wesel, Germany) under microaerobic conditions (5%  $\text{O}_2$ , 10%  $\text{CO}_2$ , 85%  $\text{N}_2$ ). The incubation temperature was  $41.5 \pm 1^{\circ}\text{C}$ . For species confirmation of the field isolates, a multiplex PCR assay targeting the 16S rRNA, *mapA*, and *ceuE* genes was performed (17).

**Inactivation and culture-based enumeration of *Campylobacter* cells.** To determine the optimal temperature for heat inactivation, approximately  $10^6$  cells were suspended in 1 ml 0.9% NaCl and exposed to  $60^{\circ}\text{C}$ ,  $65^{\circ}\text{C}$ ,  $70^{\circ}\text{C}$ , and  $75^{\circ}\text{C}$  for 15 or 20 min in a water bath (GFL, Burgwedel, Germany), respectively. The killing efficiency was checked by plating 100- $\mu\text{l}$  aliquots on at least 10 agar plates. Killing was assumed to be effective in the absence of visible growth after 48 h. Thus, a 15-min inactivation at  $70^{\circ}\text{C}$  was the best temperature-time combination completely inhibiting bacterial growth and was chosen for further experiments. Viable and nonviable cells were mixed subsequently in 1:1, 1:10, 1:100, and 1:1,000 ratios, as required. For the adjustment of *Campylobacter* cell concentrations and enumeration, four to five colonies were selected from an agar plate, transferred into a tube containing 0.9% saline solution, and adjusted to an 0.5 McFarland turbidity standard. The suspension was diluted in a 10-fold dilution series (up to  $10^{-7}$ ), and ten 100- $\mu\text{l}$  aliquots of each dilution step were streaked on CCDA plates, followed by 48 h of incubation at  $41.5 \pm 1.0^{\circ}\text{C}$  under microaerobic conditions. CFU were counted from agar plates containing 1 to 300 colonies, and the weighted average was used for the calculation of cell counts.

**EMA and PMA treatment of samples.** The intercalating dyes EMA (Invitrogen, Darmstadt, Germany) and PMA (VWR, Darmstadt, Germany) were dissolved in 20% dimethyl sulfoxide (DMSO), stored at  $-20^{\circ}\text{C}$  until needed, and added to samples to final concentrations of 10  $\mu\text{g}/\text{ml}$  (23.81  $\mu\text{mol}/\text{liter}$ ) EMA and 100  $\mu\text{g}/\text{ml}$  (238.10  $\mu\text{mol}/\text{liter}$ ) EMA or 25.55  $\mu\text{g}/\text{ml}$  (50  $\mu\text{mol}/\text{liter}$ ) PMA and 51.10  $\mu\text{g}/\text{ml}$  (100  $\mu\text{mol}/\text{liter}$ ) PMA, respectively. These concentrations have previously been reported to be suitable for quantification of viable cells (9, 18–20). Samples were incubated for 15 min in the dark at  $22 \pm 1^{\circ}\text{C}$  under frequent shaking before being placed in an iced cooling box. Subsequently, the tubes were exposed for 15 min to a 500-W halogen lamp light source (Düwi type R7s; REV Ritter, Mömbris, Germany) with 20-cm spacing from the lamp to photoinduce cross-linking of the intercalating dye.

**DNA isolation and qPCR experiments.** Genomic DNA was extracted by using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For quantitative real-time PCR (qPCR) detection of *Campylobacter*, the commercially available SureFood Pathogen *Campylobacter* Plus LC kit (Congen, Berlin, Germany) was used. This kit detects the three major food-borne species *C. jejuni*, *C. coli*, and *Campylobacter lari* by targeting the 16S rRNA gene and amplifying a 287-bp fragment. Each real-time PCR was performed in a 25- $\mu\text{l}$  volume containing 18.6  $\mu\text{l}$  *Campylobacter* Plus LC reaction mix, 1.1  $\mu\text{l}$  fluorescence detection setup (FDE), 0.3  $\mu\text{l}$  *Taq* polymerase, and 5  $\mu\text{l}$  of template DNA. PCR conditions were 60 s at  $95^{\circ}\text{C}$ , followed by 45 cycles of  $95^{\circ}\text{C}$  for 10 s and  $60^{\circ}\text{C}$  for 15 s. All assays included positive, negative, extraction, and internal amplification controls. The qPCR experiments were carried out with a LightCycler 480 system (Roche Diagnostics, Mannheim, Germany), and the instrument software was used to define the threshold cycle ( $C_T$ ) values using the second derivative method. Three independent experiments were performed.

**Determination of the signal reduction from nonviable cells.** Tenfold dilution series of heat-inactivated *C. jejuni* DSM 4688 starting from  $2.34 \times 10^7$  to  $2.99 \times 10^7$  cells were treated with 10  $\mu\text{g}/\text{ml}$  EMA, 100  $\mu\text{g}/\text{ml}$  EMA, 25.55  $\mu\text{g}/\text{ml}$  PMA, and 51.10  $\mu\text{g}/\text{ml}$  PMA, respectively, as described above. Subsequently, DNA was extracted and a qPCR detection was carried out. In addition, a double staining of heat-inactivated cells with EMA was performed. For this, aliquots of the serial dilutions were pretreated with EMA to a final concentration of 10  $\mu\text{g}/\text{ml}$ , and after photoinduced cross-linking of the dye to DNA and accompanied inactivation of un-

bound EMA (10), the cell suspension was immediately subjected to a second EMA staining step using the final concentration of 10  $\mu\text{g}/\text{ml}$  EMA. Further proceedings followed the standard protocol. For control purposes, nontreated aliquots of each serial dilution of heat-killed *Campylobacter* cells were used.

**Chicken samples and spiking.** Chicken leg quarters were purchased from a local supermarket and stored at  $-20^{\circ}\text{C}$  until processing. Samples were tested for *Campylobacter* prior to experiments and spiked with various amounts (CFU/ml) of viable, heat-killed, or mixed (1:10, 1:100, and 1:1,000 live/dead ratios) *Campylobacter* cells. Suspensions were spread on the surface of the chicken quarters and incubated for 20 min at room temperature. Bacteria were resuspended by shaking the chicken legs for 2 min in a sterile plastic bag containing 100 ml of 0.9% NaCl. Cell suspensions were subjected to qPCR and, after being diluted in a 10-fold dilution series, to culture-based enumeration as described above.

**Statistical analyses.** The slope ( $S$ ) of the standard curve, calculated from a 10-fold dilution series of *C. jejuni*, plotted against qPCR  $C_T$  values was used to estimate the PCR efficiency ( $E$ ) according to the formula  $E = 10^{(-1/S)} - 1$ . The LightCycler software (Roche) was used for data analysis. To calculate a relation between cell numbers calculated from real-time PCR assay results and plate counts, the Pearson correlation analysis was performed. In addition, the Bland-Altman analysis was used for method comparisons as proposed by Grouven et al. (21). For a comparison of mathematically calculated values and experimentally obtained values, the two-way analysis of variance (ANOVA) Sidak multiple comparison test was used. All statistical analyses were performed using the software GraphPad Prism6 (GraphPad Software, San Diego, CA).

## RESULTS

***Campylobacter* qPCR assay and effects of EMA and PMA treatment on qPCR amplification.** For the standard curve obtained from 10-fold serial dilutions of *C. jejuni* DSM 4688 without EMA or PMA pretreatment in relation to  $C_T$  values, the linear regression slope was  $-3.3493$  with a correlation coefficient ( $R^2$ ) value of 0.997. The PCR efficiency corresponded to 98.9%. Over a range of  $1.85 \times 10^2$  to  $1.85 \times 10^8$  CFU/ml, the curve shape was linear and the limit of quantification for this method was set as  $10^2$  CFU/ml. To investigate a shift in  $C_T$  values due to EMA or PMA sample treatment, amplification curves obtained from approximately  $1.9 \times 10^5$  to  $2.6 \times 10^6$  *C. jejuni* CFU/ml with and without the addition of EMA or PMA were compared, and results represented from three independent experiments. Control samples included DMSO at a final concentration of 2%. In the presence of either intercalating dyes or DMSO, the  $C_T$  values increased compared to untreated cells and higher concentrations of EMA or PMA resulted in a more prominent shift (Fig. 1).

**Differentiation between viable and heat-killed cells in mixed samples.** To compare the abilities of EMA and PMA to differentiate between viable and nonviable *Campylobacter* cells, different mixtures of live and heat-killed cells (1:1, 1:10, 1:100, and 1:1,000 ratios corresponding to 50%, 10%, 1%, and 0.1% viable cells, respectively) were subjected to qPCR analysis. For this, approximately  $2.6 \times 10^4$  CFU/ml viable cells or live/dead mixtures were either EMA or PMA treated or not. The difference between a  $C_T$  value obtained from a nontreated sample and the  $C_T$  value obtained from a pretreated sample was calculated and designated qPCR signal reduction as proposed by Nocker et al. (22). The signal reduction (negative value) is shown as an average of three independent qPCR runs in Fig. 2. The high shift of  $C_T$  values after 100- $\mu\text{g}/\text{ml}$  EMA sample treatment led to an exclusion of this EMA concentration from the further experimental setup. For all dyes and concentrations tested, an increasing amount of nonviable

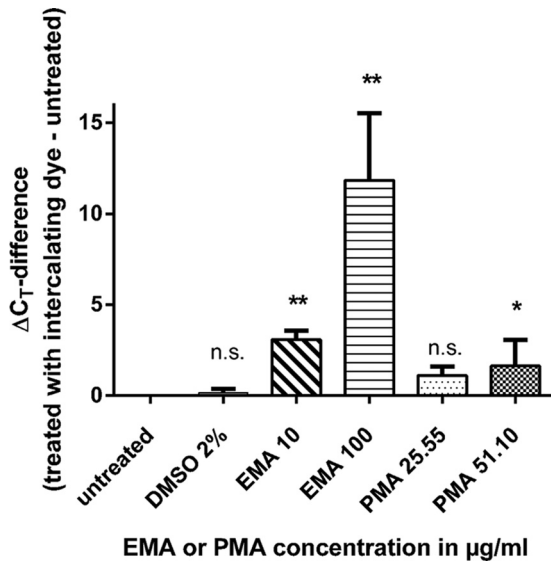


FIG 1 Differences in  $C_T$  values between qPCR results from approximately  $2 \times 10^6$  *Campylobacter* cells pretreated with either an intercalating dye or the solvent DMSO, 2% (vol/vol), in relation to untreated cells. Results are shown as averages from three independent experiments. Significant differences compared with untreated cells are indicated by asterisks (ANOVA; \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; n.s., not significant).

cells resulted in an increase in signal reduction, with the only exception being 25.55 μg/ml PMA. At this concentration, PMA did not lead to a signal reduction in mixing ratios below <10% viable cells (Fig. 2). A comparison between the experimentally deter-

mined signal reductions and the theoretical value (linear regression slope of  $-3.32$ ) by Pearson regression analysis and two-way ANOVA Sidak multiple comparison test demonstrated similar equations after pretreatment with 10 μg/ml EMA (slope,  $-3.27$ ) or 51.10 μg/ml PMA (slope,  $-2.97$ ) without significant differences between the values, whereas the use of PMA at a concentration of 25.55 μg/ml resulted in a remarkable deviation (slope,  $-1.24$ ) from the mathematically expected value.

**Limits of the EMA or PMA signal reduction from nonviable cells.** Experiments using serial dilutions of approximately  $10^7$  to  $10^2$  heat-inactivated cells demonstrated a sufficient signal reduction (defined as rounded  $C_T$  value of  $>35$ ) for the dyes and concentrations tested (10 μg/ml EMA, 25.55 μg/ml PMA, and 51.10 μg/ml PMA) if a limit of approximately  $10^4$  nonviable cells was not exceeded (Fig. 3). Signals from samples containing  $10^5$  cells were adequately inhibited only by PMA, whereas larger amounts of heat-inactivated cells ( $>10^6$ ) remained detectable independently of the dye and concentration used. However, the signals from all heat-inactivated samples were reduced considerably (on average, 5.51 to 9.85  $C_T$  values) compared to the nontreated controls. A double staining with EMA clearly improved the signal reduction and resulted in  $C_T$  values above the defined detection limit of  $>35$  (Fig. 3), if fewer than  $10^6$  inactivated cells were analyzed.

**Standard curves using EMA and PMA.** Standard curves were repeated three times independently in the presence of either of the intercalating dyes EMA at 10 μg/ml and PMA at 51.10 μg/ml, to investigate the constancy and the linearity of the  $C_T$  shift caused by the pretreatment of bacterial samples. In the presence of both EMA and PMA, the curves achieved high correlation coefficients

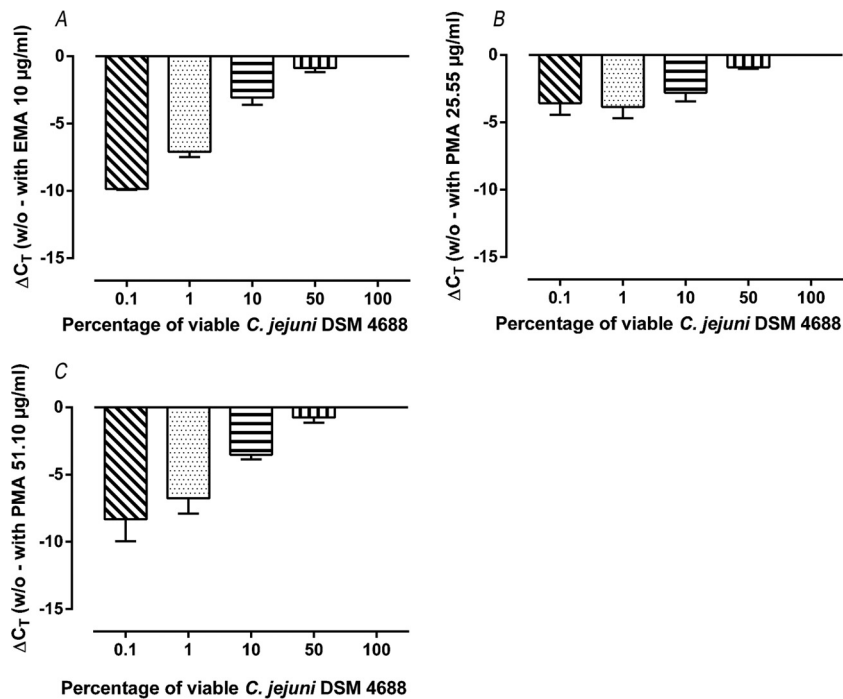


FIG 2 Signal reduction of qPCR experiments, defined as difference between  $C_T$  values obtained from a sample without pretreatment with an intercalating dye (w/o) and the  $C_T$  value derived from the respective sample pretreated with an intercalating dye. Results are shown as averages from three independent runs using 10 μg/ml EMA (A), 25.55 μg/ml PMA (B), or 51.10 μg/ml PMA (C). For all experiments, *C. jejuni* DSM 4688 was used and analyses were carried out using various percentages of viable cells.

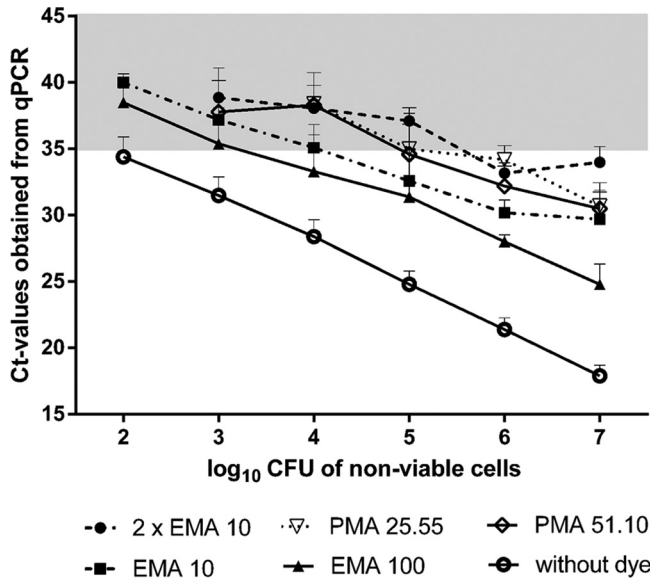


FIG 3  $C_T$  values obtained from qPCR experiments of 10-fold serial dilutions of heat-inactivated *C. jejuni* DSM 4688. Cells were pretreated with 10  $\mu\text{g/ml}$  EMA, 100  $\mu\text{g/ml}$  EMA, 25.55  $\mu\text{g/ml}$  PMA, or 51.10  $\mu\text{g/ml}$  PMA or double stained with 10  $\mu\text{g/ml}$  EMA. Results are shown as averages from three independent experiments. The absence of symbols indicates a complete inhibition of qPCR signals. The gray background represents the defined detection limit of  $>35 C_T$  values.

( $R^2$ ) of 0.997 (control, EMA) or 0.989 (PMA), respectively. As shown in Fig. 4, the  $C_T$ -shift remains constant in the presence of 10  $\mu\text{g/ml}$  EMA, and linear plots were obtained when  $C_T$  values were plotted against  $\log_{10}$  numbers of viable CFU. In the case of 51.10  $\mu\text{g/ml}$  PMA, the  $C_T$ -signal reduction depended on the concentration of CFU/ml and increasing amounts of cells/ml resulted in lower values. Although the correlation line was shifted, the slopes of the EMA and the control line were comparable ( $-3.346$  and  $-3.349$ ) and differed from that of PMA ( $-3.603$ ). Therefore, EMA (10  $\mu\text{g/ml}$ ) was used for the subsequent experiments. However, to prevent incorrect results due to the  $C_T$ -shift of pretreated

*Campylobacter* samples, an internal DNA standard was chosen from the EMA-treated cells and was used along with the adapted standard curve for the subsequent experiments in this study.

**Relation between qPCR results and microbiological cell counts.** The amounts of viable *Campylobacter* cells were quantified in parallel by qPCR and by culture-based enumeration. For this, the reference strains *C. jejuni* DSM 4688 and *C. coli* DSM 4689 as well as 16 field isolates were used. The  $C_T$  values obtained from qPCR analysis using the optimized protocol were plotted against the enumerated CFU (Fig. 5). A linear relationship ( $R^2 = 0.947$ ) was calculated, and the line had a slope of 1.029. Considering the Bland-Altman plot, the mean difference in  $\log_{10}$  CFU was  $-0.047$  with a standard deviation (SD) of 0.461. The 95% limits of agreement were 0.856 (upper limit) and  $-0.951$  (lower limit) from overall mean differences. Except for one different point located outside the detection limit, all other points were within the 95% limit, indicating that there is generally agreement between the methods (Fig. 5). Subsequently, mixtures (up to 1:1,000 ratios) of viable and nonviable cells (*C. jejuni* DSM 4688) were also subjected to a comparative Bland-Altman analysis of the two quantification methods as described above. Similar to results from viable cells, there was a linear relationship between the results obtained from the two methods ( $R^2 = 0.99$ ; slope, 1.022). The mean difference calculated from a Bland-Altman plot was  $-0.136 \pm 0.175$  (SD) CFU ( $\log_{10}$ ), and the 95% limit of agreement was 0.479 to  $-0.207$  CFU ( $\log_{10}$ ) (data not shown).

**Analysis of viable cells from spiked chicken samples.** Since poultry meat and cross-contamination in the kitchen are the major sources of human infection, the applicability of the EMA-qPCR was tested for the quantification of viable *Campylobacter* from poultry products. For this, chicken leg quarters were spiked with different quantities (range, 0 to  $10^6$  CFU/ml) of *C. jejuni* or *C. coli* field isolates or reference strains. Bacteria were rinsed out from chicken samples and investigated by EMA-qPCR and microbiological cell enumeration. It should be noted that chicken leg quarters used for these experiments were naturally contaminated with *Campylobacter* cells, ranging from  $10^2$  to  $10^3$  cells/chicken leg quarter (data not shown), and thus, a broader spectrum of cell

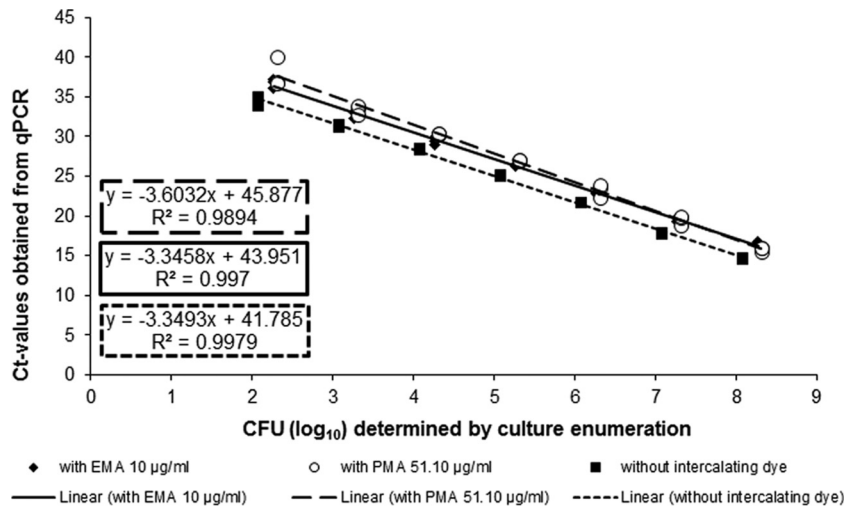


FIG 4 qPCR standard curves obtained from 10-fold dilution series of cell suspensions from *Campylobacter jejuni* DSM 4688 in the presence or absence of EMA at 10  $\mu\text{g/ml}$  or PMA at 51.10  $\mu\text{g/ml}$ . Experiments were repeated three times independently. The equation of the regression curves and the  $R^2$  values are indicated.

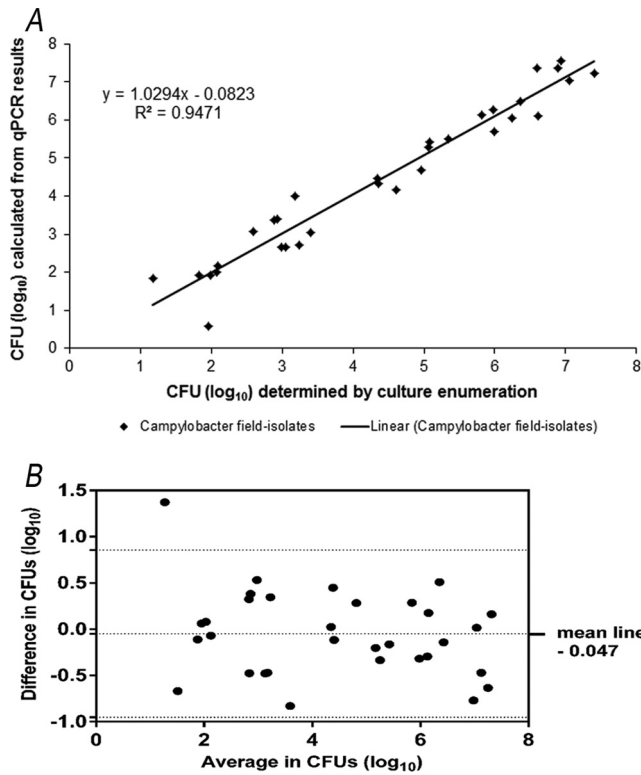


FIG 5 Correlation between *Campylobacter* cell counts of 16 field isolates plus reference strains *C. jejuni* DSM 4688 and *C. coli* DSM 4689 calculated from EMA-qPCR results and bacteriological enumeration and demonstrated by a scatter plot (A) and Bland-Altman plot for CFU determined by enumeration and qPCR results (B). In panel B, the middle line represents the mean difference of methods. Dotted lines above and below represent 95% limits of agreement.

injury and membrane damage was assumed to be present on the food samples after thawing.

For samples spiked only with viable cells, the results calculated from real-time PCR signals were in good accordance ( $R^2 = 0.95$ ; slope, 1.221) with the reference method (data not shown). Bland-Altman analysis revealed a high agreement between results from the two methods. (The mean difference was  $-0.648 \pm 0.564$  [SD] CFU [ $\log_{10}$ ], and the 95% limit of agreement was 0.458 to  $-1.754$  CFU [ $\log_{10}$ ] [data not shown]).

In a next step, mixtures with defined ratios of viable and heat-killed DSM 4688 cells (approximately  $10^5$  CFU) were used for spiking experiments. The qPCR-based quantification of *Campylobacter* without EMA detected a relatively constant number of CFU isolated from the spiked samples (mean,  $5.160 \pm 0.117$  [SD]  $\log_{10}$  CFU), independent of the percentage of viable cells (Fig. 6). In contrast, the EMA-qPCR signals ( $C_T$  values) increased as the ratio of heat-killed cells increased. This correlation is reflected in a high  $R^2$  value of 0.840 and a slope of 0.966 obtained from a plot of  $\log_{10}$  cell counts determined by EMA-qPCR versus culture enumeration (Fig. 6). Results from a Bland-Altman plot showed a low variation between the methods. The mean difference in  $\log_{10}$  CFU was 0.214. The lower and upper limits ( $\pm 0.462$  [SD] from the mean difference) of the plot were 1.119 and  $-0.691$ , respectively.

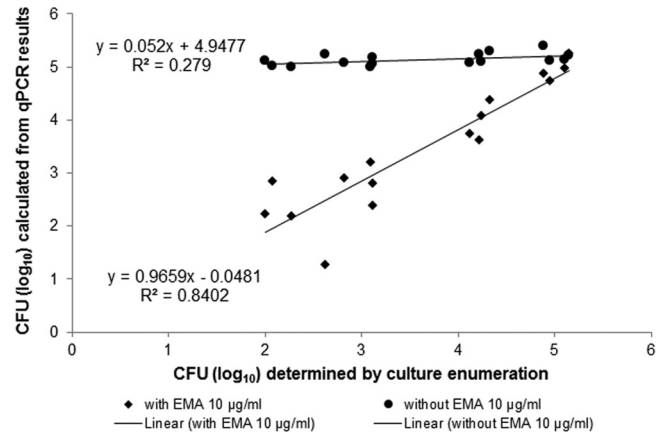


FIG 6 Scatter plot demonstrating the correlation and the difference between  $\log_{10}$  numbers of *Campylobacter* CFU/ml determined either by viability EMA-qPCR or by qPCR without EMA versus the bacterial culture method. Bacteria were rinsed from spiked chicken leg quarters, whereby the total number of spiked *Campylobacter* organisms was kept at approximately  $10^5$  cells, independent of the ratio of viable/nonviable cells. Results were shown for *C. jejuni* reference strain DSM 4688.

## DISCUSSION

The development of rapid methods for the detection and quantification of *Campylobacter* is of particular importance in food production and control and might be a step forward toward a reduction of human campylobacteriosis cases. Therefore, PCR, qPCR, or reverse transcription-PCR (RT-PCR) methods for the detection and/or quantification of *Campylobacter* have been developed and validated during recent years (5, 23–25). In addition, EMA or PMA pretreatment of bacterial samples was implemented to circumvent the amplification of DNA from nonviable cells (9, 15, 16, 26, 27).

In this study, the effects of the intercalating dyes EMA and PMA and their aptitude for a *Campylobacter* qPCR approach were comparatively analyzed. Any application of viable cells with these chemical substances or even the solvent DMSO at 2% (vol/vol) resulted in an increase of  $C_T$  values compared to untreated cells. The extent of the  $C_T$  shift depended on the concentrations tested (Fig. 1) and, as has been expected from previous studies, e.g., on *E. coli* O157:H7, *Listeria monocytogenes*, or *Enterobacter sakazakii*, was higher after EMA treatment (14, 19, 27). The characteristic of EMA of penetrating through intact cell membranes of viable cells is most likely the reason for this observation (28). Unlike some studies, e.g., on *Enterobacter sakazakii*, *Staphylococcus aureus*, *Listeria monocytogenes*, or *E. coli* O157:H7, showing no significant difference in PCR yields after PMA treatment compared to untreated cells (19, 27), we detected significantly higher  $C_T$  values ( $P < 0.05$ ) after 51.10- $\mu\text{g/ml}$  PMA application, showing that PMA in an applicable concentration penetrates membranes of viable cells as well. However, this observation may apply only to *Campylobacter* and/or selected species.

Our results indicated that the capacities of EMA-qPCR (10  $\mu\text{g/ml}$ ) and PMA-qPCR (51.10  $\mu\text{g/ml}$ ) to amplify DNA exclusively from viable *Campylobacter* cells in defined live/dead mixed ratios were comparable. The live/dead differentiation of both dyes is based on the membrane integrity of cells, and in this study, the cultivability of the bacteria on agar plates was used as a viability control. Nevertheless, it has to be taken into account that other

criteria for live/dead differentiation (e.g., energy status, metabolic activity, and RNA degradation) can be applied and other inactivation methods for cells can be used (e.g., UV exposure) and may lead to deviating findings (10, 11, 28, 29). The effective reduction of signals from dead cells was shown to be concentration dependent. More precisely, PMA at a concentration of 25.55  $\mu\text{g/ml}$  did not generate satisfactory qPCR results if mixed ratios below 10% viable cells were tested (Fig. 2B). Hence, our findings are in good accordance with studies on *Bacteroides* (30) or protozoans (31) and underline the conclusions drawn by Fittipaldi et al. (28). They reported that higher PMA concentrations may be considered to improve the signal suppression from nonviable cells, whereas lower EMA concentrations may compensate for the inability of the dye to penetrate into viable bacterial cells. Due to the considerably lower cost of EMA than of PMA and the lack of a comparative advantage, EMA could be preferred for routine diagnostic approaches and was used for most aspects of the subsequent work.

As PCR standard curves in the presence of EMA or PMA shifted toward higher  $C_T$  values, it was indicated to use a standard curve generated with EMA-pretreated cells for the purpose of a viability qPCR. Using this adapted standard curve, the EMA-qPCR provided results approximately equivalent to those of the culture-based technique as demonstrated by Bland-Altman and regression analyses, with only slight differences in assessed values depending on the strains and the live or dead/live cell mixtures that were investigated. This held true not only for *C. jejuni* and *C. coli* field isolates and reference strains from pure cultures but also for a quantitative analysis of *Campylobacter* from spiked chicken samples, implying that the two methods may be used interchangeably. It is important to mention that some chicken leg quarters, even if stored in a freezer, were naturally contaminated with approximately  $10^2$  to  $10^3$  *Campylobacter* cells (data not shown); however, this contamination and exposure of some cells to environmental stress, probably leading to a spectrum of cell injury, did not affect the correlation between culture-based enumeration and EMA-qPCR results. However, further studies on *Campylobacter* cells cultivated for longer times or exposed to environmental stresses would complete the validation of the method.

Nevertheless, experiments with serial dilutions of heat-inactivated *Campylobacter* cells demonstrated the limits of EMA or PMA pretreatment of cells. Both dyes failed to sufficiently (defined as  $C_T$  value of  $>35$ ) suppress signals from  $\geq 10^5$  nonviable cells and revealed the deficiencies of the method. These findings were in good accordance with observations recently made by Pacholewicz et al. (32), who detected an insufficient PMA effect in samples containing more than  $10^4$  dead cells. Whether an incomplete signal inhibition renders any application of an EMA- or PMA-qPCR assay impossible for routine diagnostic approaches may be interpreted in different ways. A comparison of qPCR results (genome copies) with culture-based enumeration of *Campylobacter* counts at several food processing steps in a slaughterhouse detected differences below  $10^3$ , pointing toward a relatively small amount of dead or membrane-damaged cells present on naturally contaminated food samples (32). However, the use of longer amplicon target sizes,  $>1,500$  bp, as recently proposed for a PMA-qPCR detection of *Campylobacter* (33), or a double staining with EMA, as shown in this study, is a possible option that may effectively reduce signals from membrane-damaged cells. To circumvent the disadvantages of EMA-qPCR, these options should be addressed in future *Campylobacter* qPCR research.

In conclusion, EMA as well as PMA seems to be useful for a viable/nonviable differentiation of *Campylobacter* cells from food samples containing fewer than  $10^4$  nonviable cells, but further studies are needed to overcome the limitations of these methods.

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