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Propidium monoazide does not fully inhibit the detection of dead *Campylobacter* on broiler chicken carcasses by qPCR

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ABSTRACT

A real time quantitative PCR combined with propidium monoazide (PMA) treatment of samples was implemented to quantify live *C. jejuni*, *C. coli* and *C. lari* on broiler chicken carcasses at selected processing steps in the slaughterhouse. The samples were enumerated by culture for comparison. The *Campylobacter* counts determined with the PMA-qPCR and the culture method were not concordant. We conclude that the qPCR combined with PMA treatment of the samples did not fully reduce the signal from dead cells.

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1. Introduction

Consumption of broiler meat products contaminated with *Campylobacter* is estimated to cause 20 to 30% of nine million cases of human campylobacteriosis in the European Union each year (EFSA, 2011, 2012). Most of the human infections are associated with thermotolerant *Campylobacter* such as *C. jejuni*, *C. coli* and *C. lari*. The species *C. jejuni* is by far the most important source, causing 80% of human cases of campylobacteriosis annually (EFSA, 2011). Reducing *Campylobacter* contamination along the broiler chicken supply chain could decrease human infections. The most effective intervention would be strategies at farms; however those cannot be achieved in the short term (Havelaar et al., 2007; EFSA, 2011). Therefore alternative intervention strategies should be explored in the entire broiler supply chain (Havelaar et al., 2007).

During slaughtering operations *Campylobacter* contamination on broiler carcasses fluctuate (Rosenquist et al., 2006). Interventions at the steps that contribute the most to *Campylobacter* increase on the carcasses could significantly improve safety of broiler meat (Rosenquist et al., 2003; Berrang et al., 2004). Thus, quantitative data on *Campylobacter* contamination on broiler carcasses during processing are needed, not only for evaluation of effectiveness of intervention strategies, but also for risk assessment studies.

The detection of *Campylobacter* at slaughtering operations is currently based on time and labour consuming culture methods. Molecular methods e.g. real time PCR are a rapid and high throughput alternative to traditional culture techniques. A disadvantage of real time PCR is that it does not offer any improved sensitivity over culture methods for detection and quantification of *Campylobacter* in biological samples. Another disadvantage is that dead cells may also be detected, providing unreliable information on public health risks. Live cells with intact membranes can be differentiated from dead cells by their ability to exclude DNA binding photoreactive dyes, e.g. ethidium monoazide (EMA) (Nogva et al., 2003) and propidium monoazide (PMA) (Nocker et al., 2006). Those dyes easily penetrate dead or membrane-compromised cells (Nocker et al., 2006). Inside cells dyes intercalate into double-stranded nucleic acids from cells with compromised membranes, and upon light exposure, cross-linking of the DNA occurs and its PCR amplification is inhibited (Nocker et al., 2006, 2007, 2009).

Real time PCR protocols combined with PMA treatment (PMA-qPCR) have been implemented to quantify live bacteria, e.g. *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Listeria innocua*, *Salmonella* and *Campylobacter* (Elizaquível et al., 2012; Nocker et al., 2009; Pan and Breidt, 2007; Løvdal et al., 2011; Josefsen et al., 2010; Banihashemi et al., 2012).

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The PMA-qPCR developed by Josefsen et al. (2010) was tailored to quantify live *C. jejuni*, *C. coli* and *C. lari* in chicken rinse samples obtained immediately post slaughter, thus of interest in our study. The protocol is based on the amplification and detection of a 287-bp sequence of the 16S rRNA gene of *C. jejuni*, *C. coli*, and *C. lari*. The protocol incorporates an internal amplification control (IAC) that allows monitoring of false negatives that can be caused by PCR inhibitors. In the real time PCR assay the IAC co-amplifies with the *Campylobacter* target, but both are detected with a specific probe labelled with different dyes (Josefsen et al., 2004, 2010).

The aim of this study was to evaluate the usefulness of the PMA-qPCR protocol to quantify live *C. jejuni*, *C. coli*, and *C. lari* on broiler chicken carcasses after selected processing steps in the slaughterhouse and compare PMA-qPCR results with culture. Firstly the PMA treatment was optimized and validated under laboratory conditions, including the effect of sample storage conditions.

2. Materials and methods

2.1. PMA treatment

The treatment of samples with propidium monoazide (PMA) (Biotum Inc., Hayward, CA) was implemented following the manufacturer's guidelines regarding handling of the samples during the treatment and as described by Josefsen et al. (2010) with modifications of exposure of samples to 1000 W halogen lamp at a distance of 40 cm. The effect of two variables was assessed to differentiate between live and dead cells in a culture of *C. jejuni* NCTC 12665: (i) the concentration of PMA and (ii) the time of light exposure. In the first experiment 3 concentrations of *C. jejuni* NCTC 12665 (5.3, 3.6 and 2.8 log CFU/ml) were divided in 7 aliquots that were heated at 95 °C for 5 min for killing the bacteria. One aliquot of each concentration was used as non-PMA treated control. Six remaining aliquots were treated in different combinations of PMA concentrations (20 or 50 μM) and light exposure-time (1, 2 or 3 min) (Fig. 1). In the second experiment 5 concentrations of the *C. jejuni* NCTC 12665 (4.7, 4.1, 3.4, 2.7 and 2.1 log CFU/ml) were each divided in 7 aliquots from which 4 were heated at 95 °C for 5 min for killing the bacteria and 3 aliquots were unheated live bacteria (Fig. 1). One heated and one unheated aliquot of each concentration were used as non PMA treated controls. The remaining aliquots were treated with 20 μM PMA and exposed to light (1, 2 or 3 min) to determine the effect of signal reduction from dead cells and impact of the PMA on live cells. In both experiments confirmation of the absence of live cells in the heated samples was done by culture. All samples were subjected to DNA extraction as described under Section 2.4, prior to being used as a template for the qPCR.

2.2. Analysis of inoculated samples

The PMA-qPCR protocol (Josefsen et al., 2010) for quantification of live *C. jejuni*, *C. coli* and *C. lari* in the broiler chicken carcass rinse samples was initially applied to inoculated rinse samples from broiler chicken parts purchased at a retail store. In the same experiment the impact of one day storage and storage temperature (controlled and abused) of the rinse samples on concentration of *C. jejuni* was investigated. Five chicken parts (drumstick with thigh) from different batches were rinsed vigorously by hand for 60 seconds in 125 ml peptone saline each. The rinses were inoculated with *C. jejuni* NCTC 12665 that was subcultured overnight in brain heart infusion broth to final concentrations in the samples of 3 and 6 log CFU/ml of the rinse. The inoculated samples and controls were stored for 24 h under controlled temperature (4 °C) and abused temperature that mimicked a failure of the cooling system (2 h at 4 °C, then exposed to room temperature for 2.5 h and stored again at 4 °C). Before and after storage the samples were subjected to *Campylobacter* culture on mCCDA (bioTrading, Mijdrecht, The Netherlands) for enumeration according to ISO 10272-2. In parallel the samples were subjected to PMA treatment as described by Josefsen et al. (2010), with modification of the light source and distance. After the PMA treatment the DNA was extracted and the qPCR was performed.

2.3. Analysis of naturally contaminated samples

The PMA-qPCR protocol was used to quantify live *C. jejuni*, *C. coli* and *C. lari* in rinse samples from whole broiler chicken carcasses, collected

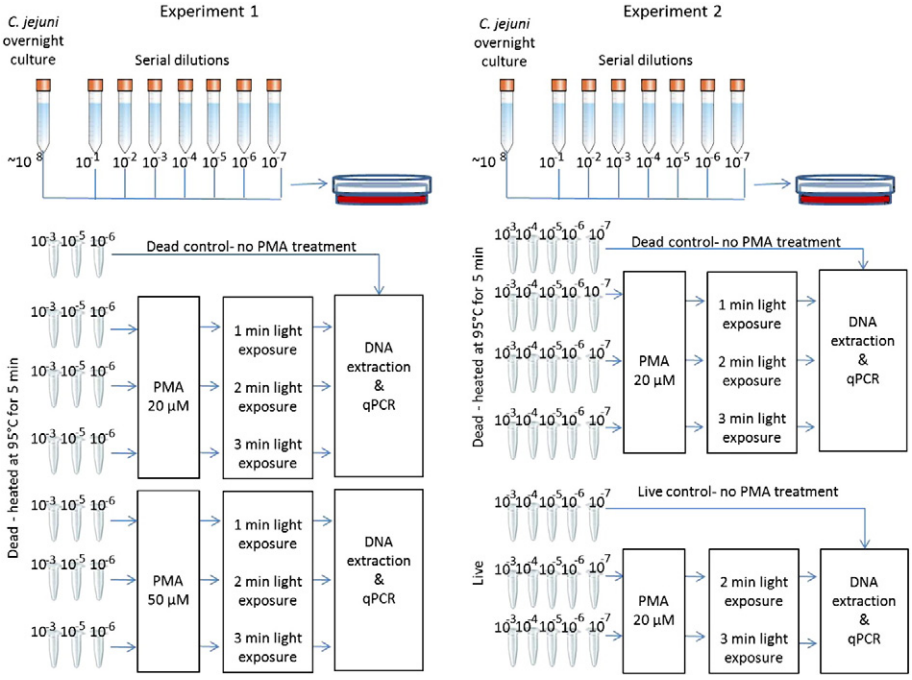

Fig. 1. Summary of experimental work set up described under point 2.1. Selected dilutions were used in the experiments. Extrapolation of counts in the dilutions resulted in the following concentrations: experiment 1 (5.3, 3.6 and 2.8 log CFU/ml), experiment 2 (4.7, 4.1, 3.4, 2.7 and 2.1 log CFU/ml). Aliquots of the selected dilutions were treated in different combinations of PMA concentration and light exposure time. Controls containing live or dead bacteria were included.

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