Federal Institute for Risk Assessment, Department of Biological Safety, National Reference Laboratory for *Campylobacter*, Berlin, Germany

Campylobacter detection along the food chain – towards improved quantitative risk analysis by live/dead discriminatory culture-independent methods

Detektion von Campylobacter entlang der Lebensmittelkette – auf dem Weg zu einer verbesserten quantitativen Risikoanalyse durch lebend/tot unterscheidende, kultivierungsunabhängige Methoden

Kerstin Stingl, Christiane Buhler, Nora-Johanna Krüger

Summary

Death, although absolute in its consequence, is not measurable by an absolute parameter in bacteria. Viability assays address different aspects of life, e. g. the capability to form a colony on an agar plate (CFU), metabolic properties or membrane integrity. For food safety, presence of infectious potential is the relevant criterion for risk assessment, currently only partly reflected by the quantification of CFU. It will be necessary for future improved risk assessment, in particular when fastidious bacterial pathogens are implicated, to enhance the informative value of CFU. This might be feasible by quantification of the number of intact and potentially infectious *Campylobacter*, impermeable to the DNA intercalating dye propidium monoazide (PMA). The latter are quantifiable by the combination of PMA with real-time PCR, although thorough controls have to be developed for standardization and the circumvention of pitfalls. Under consideration of different physiological states of the food-borne pathogen, we provide an overview of current and future suitable detection/quantification targets along the food chain, including putative limitations of detection.

Keywords: real-time PCR, metabolic state, detection methods, bacterial death, PMA, EMA

Zusammenfassung

Der Tod, wenngleich in seiner Konsequenz absolut, ist bei Bakterien nicht absolut messbar. Untersuchungen zur Lebensfähigkeit zielen auf verschiedene Aspekte des Lebens, z. B. die Fähigkeit, eine Kolonie auf einer Platte zu bilden (KbE), metabolische Eigenschaften oder die Integrität der Membran. Für die Lebensmittelsicherheit stellt das relevante Kriterium für Risikobewertung das infektiöse Potenzial dar, welches zurzeit nur teilweise durch die Quantifizierung der KbE repräsentiert wird. Insbesondere bei anspruchsvollen bakteriellen Pathogenen wird es zukünftig für eine verbesserte Risikobewertung notwendig sein, den Informationsgehalt der KbE zu erweitern. Dies könnte durch die Quantifizierung intakter und potentiell infektiöser *Campylobacter* realisiert werden, welche impermeabel für den in DNA interkalierenden Farbstoff Propidium-Monoazid (PMA) sind. Diese sind durch die Kombination von PMA mit Real-time PCR quantifizierbar, obwohl sorgfältige Kontrollen zur Standardisierung und für das Umgehen von Artefakten entwickelt werden müssen. Unter Berücksichtigung der verschiedenen physiologischen Zustände des Lebensmittel-Pathogens, geben wir eine Übersicht über die derzeitigen und zukünftigen geeigneten Detektions-/Quantifizierungs-Zielparameter entlang der Lebensmittelkette, einschließlich möglicher Detektionsgrenzen.

Schlüsselwörter: Real-time PCR, metabolischer Zustand, Detektionsmethoden, bakterieller Tod, PMA, EMA

Introduction

The microaerobic fastidious bacterial pathogen *Campylobacter* is the most frequent cause of food-borne infections in Europe (EFSA and ECDC, 2014). Due to this high frequency of infection and the underestimated infection-triggered autoimmune sequelae, the bacterium causes the highest DALYs (disability-adjusted life years) of all food-borne pathogens according to a recent presentation of the European Centre for Disease Prevention and Control (ECDC, European Commission, May 2014, unpublished). *Campylobacter* spp. are apparently successful in spreading across the world within different hosts (avian, mammalian, molluscs). In particular, *Campylobacter*-contaminated broiler carcasses comprised over 70% of the total examined carcasses, compared with a *Salmonella* Enteritidis or *S.* Typhimurium prevalence of 3.6% at community level (EFSA, 2010a). While the prevalence of *Salmonella* is decreasing owing to the control measures taken, *Campylobacter* remains a very important food-borne pathogen for which control strategies are still largely missing.

Upon exit of the intestine, the natural niche of multiplication, the bacterium rapidly loses its capacity to grow in vitro. This stress-sensitive nature of *Campylobacter* complicates the detection of the bacterium by culture-based methods. What are the most appropriate detection methods throughout the food chain? How can we use real-time PCR methods for the amelioration of quantitative detection? And most important, how to define *Campylobacter* death as negation of infectivity in order to improve quantitative risk assessment for food control?

Definition of bacterial death

For food authorities, a viable bacterium forms a colony on a nutrient agar plate. Since decades microbiologists are aware of the "great plate count anomaly" (Amann et al., 1995). Only a fraction of microbial life can be cultivated and cultivation efficiency depends on cultivation conditions and the physiological state of the target bacterium. Thus, it becomes more and more obvious that the infectious risk of fastidious bacteria can be underestimated and that for intracellular bacteria or viruses the parameter CFU is unsuitable. It is a common, but far from an absolute principle, that the more stress a bacterium is exposed to, the more likely it loses its infectious potential. The ability of pathogens to resurrect after stress exposure and transient loss of in vitro colony formation capability represents a major limiting factor of the parameter CFU for risk analysis. Hence, researchers established other means for detection of bacterial death/life (reviewed e. g. in Keer and Birch, 2003; Sträuber and Müller, 2010; Davey, 2011; Hammes et al., 2011) and workshops were launched addressing the question of how to interpret these alternative parameters, summarized by the famous question „How dead is dead?" (http://www.hdid-conference.de/).

It is obvious that the term death, although absolute in its consequence, is not represented by an absolutely measurable parameter for bacteria. Four main stages for the characterization of bacterial viability/death were defined, corresponding to reproductive growing cells, metabolically active cells, cells with an intact membrane and permeabilized cells (Nebe-von-Caron et al., 2000). Detection methods quantifying the number of viable

bacteria target these different aspects/stages of life; (i), the capability of a single cell to multiply in vitro corresponds to the number of CFU under conditions quite distinct from those in vivo (ISO 10272-2, 2006); (ii), the amount of RNA per cell measures the magnitude of current transcription/translation, which varies depending on the growth phase of the bacteria (Schaechter et al., 1958); (iii), the metabolic state of a bacterium, which is reflected by the magnitude of the proton motive force or the concentration of the energy source adenosine triphosphate at the moment of measurement (Tholozan et al., 1999); (iv), the integrity of the bacterial membrane (Fittipaldi et al., 2012), i. e. the number and size of membrane leakages, which also present some variations. Therefore, each time the term "viable" is used, it has to refer to a specific context. It has fundamental practical implications for the assessment of the risk for infection, e. g. that the seemingly clear-cut parameter CFU masks infectious potential.

The question arises how *Campylobacter* die under "physiological" conditions on chicken products and how to visualize this "physiological death". The parameter CFU apparently underestimates the infectious potential, in particular after stress exposure of the bacterium, e. g. on retail products (see below). Quantitative determination of specific mRNA or 16S rRNA matched well with CFU in chicken faeces (Bui et al., 2012). Hence, in this study reverse transcriptase PCR did not enhance the informative value of CFU. To our mind, the most likely physiological death of the microaerobic bacterium on chicken products is caused by oxidative stress. It was exemplary shown that oxidative stress decreased *Campylobacter* CFU and, concomitantly, the amount of a stress-sensitive oxidoreductase (Yamasaki et al., 2004). Moreover, cold stress slows down enzymatic functions, like katalase (Hazeleger et al., 1998) and superoxide dismutase, which are implicated in the combat against oxidative stress (Flint et al., 2014). Note that the intrinsic metabolism, which also produces putatively poisonous oxygen species, is also slowed down at cold temperatures, which might compensate for lower activity of essential enzymes involved in stress response under such conditions. However, with time oxidation of lipids and proteins cannot be prevented nor restored, slowly enhancing loss of cell functions and loss of membrane integrity. We showed recently that artificially killed *Campylobacter* using 5% H_2O_2 led to complete loss of CFU (> 6 log), maintenance of the DNA inaccessible to DNase (absence of bacterial lysis), and access of DNA intercalating dyes to the cytoplasm (loss of membrane integrity) (Krüger et al., 2014). In contrast, transient de-energization of *Campylobacter* led to transient loss of membrane polarization but did not lead to any decrease in CFU after re-energization. In conclusion, a pragmatic way of defining bacterial death for food control would be the monitoring of membrane integrity rather than the metabolic state of the bacterium. We suggest to determine "intact and potentially infectious units" (IPIU) as a quantitative parameter measuring the number of bacteria with an intact membrane impermeable to propidium iodide (and its analogue propidium monoazide), previously applied in live/dead discrimination (Boulos et al., 1999; Giao et al., 2009). Bacteria with intact membranes are principally capable of metabolic activity and repair of sublethal damage. Hence, it was previously suggested that in product safety applications, death would ideally be defined by loss of membrane integrity (Nebe-von-Caron et al., 2000).

Principles of real-time PCR quantification in combination with live/dead discrimination

Live/dead discrimination using DNA intercalating dyes is based on differences in membrane permeability of viable and dead bacteria (for a review see Fittipaldi et al., 2012). These permeability differences lead to the selective intercalation of monoazide dye analogues into cytoplasmic DNA located in membrane-compromised (dead) bacteria. Upon DNA intercalation of these dyes, covalent binding of the molecules to DNA is accomplished by light-induced crosslinking, inactivating DNA for subsequent PCR amplification. In an ideal scenario, the total PCR signal stems from viable cells, whereas the dead cell signal is completely absent. Plenty of studies revealed that this ideal scenario differs from reality and we will discuss in the following crucial parameters for maximal live *Campylobacter* detection with concomitant minimal signal from dead cells.

Dye exclusion from live bacteria
In order to get a clear picture about the choice of a suitable quantitative live/dead discriminatory dye, the principles of dye exclusion from live bacteria have to be uncovered. If exclusion of the dye is based on active efflux of the dye from the bacterium, not only integrity of the membrane but also the metabolic state of the bacterium will be determined. The latter depends on the growth phase and stress conditions and leads to underestimation of intact and potentially infectious units. It is desirable that the dye indicator is passively excluded from (transiently inactive or less active) cells. For this reason, we performed membrane permeability assays with propidium iodide (PI) and its analogue propidium monoazide (PMA) or ethidium bromide (EtBr) and its analogue ethidium monoazide (EMA), intercalating dyes used for the quantitative detection of viable *Campylobacter* (Rudi et al., 2005; Josefsen et al., 2010; Pacholewicz et al., 2013; Seinige et al., 2014). When we abolished the proton motive force of *Campylobacter* by addition of an uncoupler of the proton gradient across the inner membrane or when we selectively inhibited efflux transporters, EtBr significantly entered the bacterium in a time- and concentration-dependent manner

(Krüger et al., 2014). Release of uncoupler or efflux transport inhibitor completely restored the capability to form a CFU, thereby, demonstrating a transient non-detectable state of *Campylobacter* (EtBr entry) with full bacterial resurrection after stress release. In untreated control cells, we observed marginal entry of EtBr into exponentially grown *Campylobacter* but considerable entry into stationary grown cells, respectively. In contrast, PI stayed passively out of the bacterium, irrespective of the metabolic state and/or treatment of the cells and was a reliable indicator of death-related membrane leakage. The respective data for the photo-crosslinkable analogues are summarized in Table 1. Depending on the energy status of the cell, variable influx of EMA into *Campylobacter* was observed, as detected by reduction of PCR signal. This influx was most pronounced in buffer without nutrients (de-energized state of the bacterium) and for stationary phase bacteria that are less metabolically active. In contrast and in agreement with our observations obtained with PI, PMA was passively excluded from all viable bacteria, irrespective of the energy state or the growth phase. Also for other bacterial species, it was acknowledged that PMA has advantage over EMA concerning more efficient discrimination between viable and dead bacteria (Nocker et al., 2006; Loozen et al.; 2011; Yanez et al., 2011).

In a recent study, the authors concluded that EMA was a promising rapid method for the diagnostic application in *Campylobacter* quantification (Seinige et al., 2014). The authors correlated EMA-qPCR results of spiked "reference *Campylobacter*" with laboratory-cultivated "field strains" of comparable physiological state. However, when EMA signal reduction from viable cells is standardized using laboratory strains under optimal energetic conditions, it is likely that the number of intact and potentially infectious bacteria (with unknown metabolic states and unknown history of stress exposure) on food samples are underestimated. Moreover, the authors also observed a drastic dependence of EMA concentration on entry into *Campylobacter*, leading to huge reduction of viable counts when a much higher concentration of 100 µg/ml EMA was used. Insufficient differentiation of live and dead *Campylobacter* by EMA was also demonstrated previously (Flekna et al., 2007). Also He and Chen (2010) reported toxic effects on viable cells in the presence of 100 µg/ml of EMA, although they obtained promising results for the detection of coccoid *Campylobacter* with lower EMA concentrations. In contrast, Rudi et al. (2005) had observed marginal dye influx into viable *Campylobacter* using 100 µg/ml of EMA. How can these divergent data from different laboratories be explained? The actual concentration of dye at the bacterial membrane is largely influenced by the heterogeneous matrix, and also different temperature-time combinations during dye incubation significantly contributed to the amount of EMA entry into viable *Campylobacter* (Krüger et al., 2014). After incubation of *Campylobacter* with EMA on ice, we indeed observed much less entry of the dye into viable bacteria. However, the signal reduction of dead *Campylo-*

TABLE 1: *Decrease of qPCR detection (Δct) of live Campylobacter by use of intercalator dyes*

Campylobacter growth phase	Condition	Energy state	EMA influx		PM influx	
				Δct		Δct
Exponential	Chicken rinse			1.1 ± 0.8		
Exponential	Buffered peptone water			1.9 ± 0.4		0.3 ± 0.3
Stationary	Chicken rinse			4.0 ± 0.6		
Stationary	Buffered peptone water			6.0 ± 2.5		
All	PBS			8.8 ± 2.4		0.4 ± 0.6
All	PBS + Inhibition of active efflux-transporters			n. d.		0.9 ± 0.5
All	PBS + Transient depletion of proton-motive force			n. d.		0.6 ± 0.4

PMA is passively excluded from intact *Campylobacter*, while EMA enters live bacteria in an energy-dependent manner. Δct-values (± 20 µM dye) after 15 min of incubation at room temperature are depicted from Krüger et al. (2014). Δct-values for higher PMA concentrations (up to 100 µM) were comparable to those observed for 20 µM.

bacter with EMA was not satisfactory under the same temperature-time conditions in the presence of chicken rinse (Krüger et al., 2014). It has to be noticed that the way of inactivation of bacteria for the production of a dead cell standard can also lead to variation of DNA loss from bacteria (Krüger et al., 2014) and the degree of intercalator dye-qPCR reduction (Rudi et al., 2005).

In conclusion, the fact that entry of EMA into viable *Campylobacter* is concentration-, matrix-, temperature- and time-dependent, reproducible processing of a large number of samples in routine laboratories is complicated, while, in addition, appropriate reference standards are missing. Using PMA, these uncertainties concerning the current metabolic state of the target viable bacteria and/or moderate differences of processing time and temperature are negligible, since PMA is passively excluded from intact and potentially infectious units in a reproducible and robust manner.

Maximal reduction of the residual signal from dead cells

In addition, maximal reduction of the residual signal from dead cells is crucial. We do not further address EMA for reasons explained above but point out that the same principles are valid for both photo-crosslinkable dyes concerning residual dead cell signal. The magnitude of signal reduction of dead bacteria is based on the concentration of the dye at the bacterial membrane, the size and amount of membrane leakages per bacterium, the temperature and the target size used in the real-time PCR assay. Entry of PMA into membrane-compromised bacteria is a diffusion-limited process. Hence, the amount of PMA reaching the cytoplasmically located DNA within a given time period is not only dependent on the concentration gradient at the bacterial membrane but also on temperature. Consistently, higher incubation temperature resulted in improved efficiency of PMA to reduce false-positive signal from dead bacteria (Nkuipou-Kenfack et al., 2013). Temperatures slightly below or equal than growth temperature (in order to maintain viability of the target live bacteria) will lead to higher diffusion of the dye through membrane leakages into dead bacteria. The actual concentration of the dye at the bacterial membrane is influenced by the amount of organic matter, like proteins, free DNA and/or tissue present in the food matrix. In order to lower at least soluble compounds, centrifugation and resuspension in phosphate-buffered saline prior to staining with PMA improved dead cell signal reduction (Krüger et al., 2014). Moreover, the size of the target sequence used for real-time PCR detection defines the magnitude of dead cell signal reduction. In principle, the longer the target fragment, the higher the probability of PMA to intercalate into the target sequence hindering PCR amplification. Likewise, the magnitude of dead cell signal inactivation increased with amplicon length (Contreras et al., 2011). An optimal amplicon length provides sufficient PCR efficiencies with maximal dead cell signal reductions. The 287 bp amplicon of the 16S rRNA gene targeted by real-time PCR was published as official method in the framework of the § 64 of the German Food and Feed Code (LFGB, 2013) (Lübeck et al., 2003; Josefsen et al., 2004). In most real-time PCR applications, a target length of ≤ 100 bp is used for optimal PCR performance. Although the chosen target is longer, complete elimination of rest signal from dead cells was not feasible (Pacholewicz et al., 2013). However, this target was used for the quantitative detection of viable *Campylobacter* in different chicken matrices in excess of 2.5–3 log dead bacteria (Krüger et al., 2014).

The need for an appropriate sample process control for quantification

Real-time PCR determines the number of target sequences, i. e. chromosomal copies within the sample of interest. Although the number of chromosomes can significantly deviate from one per bacterium, in particular in fast-growing organisms (Ferullo et al., 2009), only a 1.3-fold relative difference of chromosomal copy number per bacterium estimated after DNA extraction was observed for *Campylobacter* grown to exponential versus stationary phase under our conditions. Thus, the number of chromosomal copies well matched the number of cell counts for a large variation of growth conditions. It has to be kept in mind that the correlation of cell count with CFU is highly variable and depends on the bacterium's physiological condition (Krüger et al., 2014) and the growth medium used (Rodgers et al., 2012). Therefore, it is unrealistic that one *Campylobacter* corresponds to one CFU detected, unless the most active bacteria are identified by flow cytometry analysis and subsequently selected by fluorescence-activated cell sorting (Matsuoka et al., 2014). Moreover, aggregation of bacteria can decrease the number of CFU. Hence, quantitative PCR results should be translated into chromosomal number or into cell counts, not CFU. For practical implementation, the number of cell counts can be estimated from the optical density, with $OD_{600nm} = 0.2$ corresponding to approximately 10^9 cell counts per ml (Krüger et al., 2014).

The yield of DNA preparations from bacteria differs according to the extraction methods applied. Even if a method is routinely applied, the amount of total organic matter of the heterogeneous matrices can vary considerably, contributing to alterations in DNA extraction efficiency. In addition, PMA diffusion in "dead" cells and crosslink efficiency to DNA should be monitored for each sample. Oxidative stress treatment or controlled heat treatment of *C. jejuni* with concomitant monitoring of bacterial lysis will be useful for laboratories to generate their own "dead cell standard". In future, a distinct number of membrane-compromised bacteria with defined membrane permeability properties should be detectable by real-time PCR using an independent target sequence. This internal sample process control (ISPC) will lead to reliable quantitative detection of *Campylobacter* by constant monitoring of all relevant parameters. The distinct number of cell counts without PMA treatment would serve as quantification standard for DNA extraction. The defined membrane permeability properties would be helpful in referring to the same degree of membrane leakage ("death") and could monitor the efficiency for dead cell signal reduction throughout the procedure. In consequence, different laboratories would be enabled to obtain reproducible quantitative results, using slightly deviating methods and various undefined matrices.

Suitable detection of *Campylobacter* along the food chain

Given the above mentioned fact that detection of viable *Campylobacter* is hampered by inefficient quantitative

culturability after stress exposure, we noticed that there is much confusion about the choice of a suitable detection method for different samples along the food chain. In Table 2 we summarized the different food chain stages of chicken, including an estimate of the concentration of *Campylobacter* expected to be found, the assumed physiological condition of the bacterium (stressed, non-stressed) and the resulting recommended parameter/method for reliable qualitative and/or quantitative detection.

Primary production

Upon colonization of the host intestine, *Campylobacter* are constantly shed and can be detected in faecal droppings. Once introduced into a flock, the bacterium can spread within a few days (van Gerwe et al., 2005). Concerning the concentration of *Campylobacter*, caecal contents exhibit the highest *Campylobacter* load, ranging between 10^6 and 10^8 CFU/g (Musgrove et al., 2001; Stern et al., 2005; Reich et al., 2008; Rodgers et al., 2012). Storage for 72 hours at cold temperatures led to a slight reduction in the number of culturable bacteria, still leading to a highly reliable detection of the bacterium by growth on agar plates (Rodgers et al., 2012). Since faecal and caecal samples contain very high concentrations of the bacterium, enrichment, which bears the risk of overgrowth of resistant competitive flora, will be omitted in the modified ISO 10272-1C by direct plating on mCCDA (Technical Advisory Group CEN/TC 275/WG 6/*TAG* 19, personal communication).

Outside the intestine, the microaerobic and thermophilic bacterium is primarily confronted with oxidative, cold and drought stress. Hence, the efficiency of *Campylobacter* detection in faecal droppings depends on the time period after faecal exit from the intestine. Consistently, the cultural detection of *Campylobacter* decreased

with time (Bui et al., 2012; Ahmed et al., 2013). While quantitative cultural detection is feasible from fresh faecal droppings, *Campylobacter* are insufficiently detected after long-term exposure to cold, oxygen and drought. In consequence, sampling of already desiccated faecal droppings, which can occur by using boot socks, might lead to a lower detection of *Campylobacter* CFU. However, detection of DNA by real-time PCR is a rapid method and reliably indicates flock colonization in primary production, irrespective of the sample type. Since faecal shedding of *Campylobacter* correlates with active colonization and multiplication of the bacterium within the intestine of chicken, evidence of bacterial viability during the detection assay is dispensable for the interpretation of the *Campylobacter* status of the flock. Importantly, DNA extraction from faeces has to be checked for PCR inhibitors by adding an internal amplification control. Common detection limits range around 10^3–10^4 bacterial counts/g faeces (Rodgers et al., 2012; Garcia et al., 2013; Janzer et al., 2014) and can be explained from the detection limits of real-time PCR of around ten copies per reaction and the fact that only a fraction of the extracted DNA can be analysed per assay without PCR inhibition. However, since the concentration of *Campylobacter* in faeces significantly exceeds 10^4 bacterial counts per g in most cases, detection limits are negligible. Note that if numbers of 1–5 CFU/g are reported as detection limits in real-time PCR applications, either this number refers to the number of initially spiked CFU before enrichment or the number of CFU corresponds to a higher number of bacterial counts, since not every bacterium forms a CFU on the agar plate (as discussed above).

Slaughterhouse

Concentrations of *Campylobacter* within caecal or faecal contents of chicken are approximately 3 log

**TABLE 2:** *Overview of suitable Campylobacter detection along the chicken food chain*

Stage of food chain	Sample type	Expected concentrations of *Campylobacter* per g[1,2]	Exposure time, stress condition of *Campylobacter*	Suitable parameter/s for risk assessment	Explanatory statement
Primary production	Caecal content	10^6–10^8 (++)[a,4]	short-term, cold/oxygen	CFU, DNA	Fecal shedding of *Campylobacter* indicates colonization of the flock; intrinsic evidence for bacterial viability and, thus, DNA detection sufficient for the determination of the flock status
	Fresh faecal droppings	10^5–10^7 (++)[b]	short-term, cold/oxygen	CFU, DNA	
	Faecal droppings (wet)	10^5–10^7 (+)[c]	medium-term, cold/oxygen	growth (yes/no), DNA	
	Boot socks (incl. dried faeces)	10^4–10^6 (+ or ±)[d]	long-term, cold/oxygen/drought	DNA, growth (yes/no)	
Slaughterhouse	Carcass skin	10^1–10^4 (++)[e]	short-term, cold/oxygen	CFU, IPIU[3]	Fresh contamination of carcass surface; reasonable detection by CFU unless stress has been exerted by e.g. decontamination
	Carcass skin after decontamination (e. g. lactic acid)	$\leq 10^1$–10^4 (+ or ±)[f]	short-term, cold/oxygen/acid	IPIU[3], growth (yes/no)	
Retail	Fresh chicken	10^1–10^4 (+)[g]	long-term, cold/oxygen	IPIU[3], growth (yes/no)	Only qualitative culture-dependent detection reasonable; IPIU eligible for quantification
	Frozen chicken and meat preparations	$< 10^1$–10^4 (±)[h]	long-term, cold/oxygen/freezing and/or spice	IPIU[3]	

[1] Numbers of total cell counts (not CFU) estimated on the basis of a, (Musgrove et al., 2001; Stern et al., 2005; Reich et al., 2008; Rodgers et al., 2012); b, (Stern and Robach, 2003; Ahmed et al., 2013; Berghaus et al., 2013); c, (Bui et al 2012; Ahmed et al., 2013); d, (Cox et al., 2001; Berghaus et al., 2013); e, (Stern and Robach, 2003; Rosenquist et al., 2006; Reich et al., 2008; EFSA, 2010b; Stingl et al., 2012; Pacholewicz et al., 2013); f, (EFSA, 2011); g, (Callicott et al., 2008; Baumgartner and Felleisen, 2011; Stingl et al., 2012); h, (Callicott et al., 2008; Habib et al., 2008; Baumgartner and Felleisen; 2011).

[2] In brackets, estimation of the portion of non-stressed bacteria capable to be detected as CFU; ++, high amount of non-stressed bacteria; +, medium amount of non-stressed bacteria; ±, most bacteria stressed (and not able to form a CFU).

[3] IPIU, intact and potentially infectious units detected by qPCR in combination with PMA

[4] Note that even higher concentrations were recently found (median of 10^9/g; Vidal, A., personal communication)
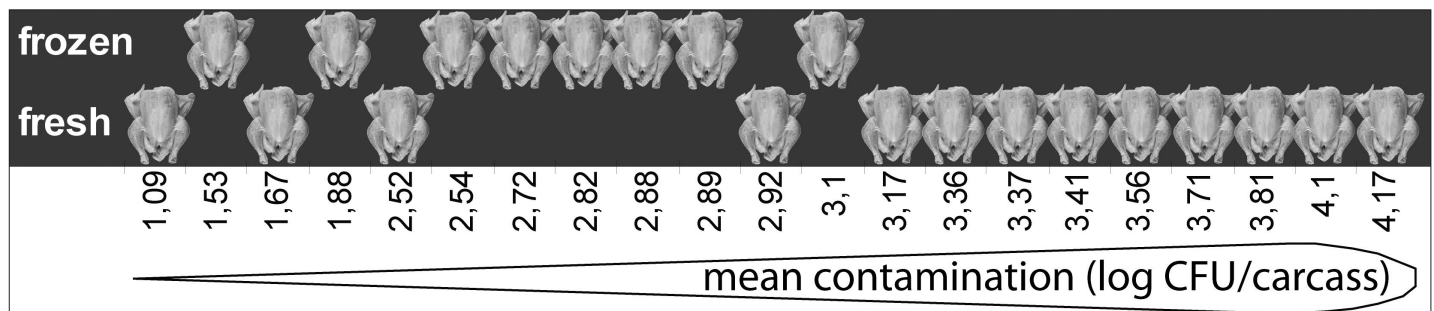
FIGURE 1: *Putative underestimation of the infectious potential of culturable number of Campylobacter from frozen chicken. CFU of Campylobacter on chicken carcasses (fresh versus frozen), which were directly implicated in human campylobacteriosis cases in Iceland, were taken from the study of Callicott et al., 2008. Frozen chicken implicated in human campylobacteriosis appeared to exhibit lower CFU than fresh chicken. Note that the overall number of data is limited and that the number of infected humans (which is not known but might be deduced from the number of collected and matching isolates) is not considered.*

higher compared to those of *Salmonella* (Berghaus et al., 2013). In the process of slaughtering, in particular during defeathering and evisceration, faecal leakage leads in turn to relatively high contamination of the carcass with *Campylobacter* (Rosenquist et al., 2006; Pacholewicz et al., 2013), leading to common contamination levels of 10^1–10^4 CFU per g skin for carcasses after chilling (Stern and Robach, 2003; Rosenquist et al., 2006; Reich et al., 2008; EFSA, 2010b; Stingl et al., 2012; Pacholewicz et al., 2013). Since *Campylobacter* just exited the intestine, the bacterium on these fresh carcass skin samples is reasonably quantified using the standard ISO 10272-2:2006 enumeration method. A quantitative microbiological criterion of 500–1000 CFU/g skin is currently discussed by the EU commission and planned to be set at the slaughterhouse (EFSA, 2011).

Decontamination of carcasses is already implemented in some countries as control options, while the European Union still maintains a reluctant attitude towards those measures. Decontamination efficiencies, summarized in a recent EFSA opinion are commonly evaluated on the basis of CFU (EFSA, 2011). It remains to be elucidated, to which extent these procedures also lead to significant reduction in IPIU, using PMA-based real-time PCR.

Retail
Due to the remarkable decrease in growth capabilities of stressed bacteria, there is a considerable gap between the detection limit of *Campylobacter* by cultivation and its infectious potential. No reduction of *Campylobacter* prevalence but a significant quantitative reduction of the number of culturable *Campylobacter* was observed comparing German broiler carcasses sampled at slaughterhouse with fresh meat at retail (Stingl et al., 2012). Loss of skin (e. g. breast filet) or freezing of a part of the products might have contributed to a lower quantitative contamination load of viable bacteria on the retail product (Baumgartner and Felleisen, 2011). However, with respect to chicken retail products presenting the main source for *Campylobacter* infection, the residual intact and potentially infectious units (IPIU) of *Campylobacter* on chicken meat must be considered a sufficient threat for human infections.

Freezing is considered to be a physical decontamination process, leading to a two log reduction of *Campylobacter* CFU after three weeks of freezing (EFSA, 2011). The study of Callicott and colleagues (2008) directly matched broiler batches with human campylobacteri-

osis cases in Iceland on the basis of *flaA* genotypes. This study is exceptional, since only domestically acquired campylobacteriosis cases were analysed and a high percentage of all broiler lots were sampled, since the production industry is small and delimited. Genetically matching isolates within a time frame of two weeks between broiler processing and human infection onset were defined as implicated in human disease. As result the implicated product lots of fresh meat had a significantly higher contamination level than the non-implicated lots. For frozen retail products, implicated lots were significantly larger than non-implicated lots (Callicott et al., 2008). We asked whether the mean contamination level of implicated fresh and frozen lots differed significantly using the quantitative data from the latter study, eventually indicating underestimation of the infectious potential of *Campylobacter* by quantification of CFU. As expected, the implicated frozen products had an overall lower contamination level as measured by CFU than the fresh products (Fig. 1). However, the conclusion is limited due to the restricted number of broiler batches with quantitative contamination data directly matched to human cases in this study (n = 21, Callicott et al., 2008; Fig. 1). Besides, the number of identified human cases matching the 13 fresh broiler lots was higher (n = 45) than for frozen lots (n = 8), although quantitative data of eight further frozen implicated broiler lots were missing, which genetically matched 18 of the 45 human cases connected with fresh meat. Nevertheless, although freezing does reduce the number of CFU, the infectious potential of frozen products might not be reduced to the same extent and can significantly be underestimated. This issue will have to be addressed in more detail in future studies with enhanced informative value derived from IPIU quantification using PMA-based qPCR.

Conclusion

The parameter CFU, apparently clear-cut in its quantitative interpretation, can mask the infectious risk when applied to fastidious microorganisms after stress exposure. However, for fresh samples, e. g. from the slaughterhouse, CFU is a reasonable parameter for the contamination level of *Campylobacter* on carcasses and might be targeted in food control by setting an appropriate microbiological criterion. For the improvement of the quantitative risk assessment along the complete

food chain, it is of high interest to define bacterial death by a reliable parameter other than CFU. We suggest to quantify intact and potentially infectious units (IPIU), pragmatically defined as bacteria with an intact membrane impermeable to PMA. As a prerequisite, an internal sample process control (ISPC) has to be generated, which can be detected independent of the target *Campylobacter*. This ISPC will define "bacterial death" and absolute number of cell counts by providing a distinct number of membrane compromised bacteria with defined permeability properties. We are aware that the implementation of IPIU in food legislative is not subject to immediate acceptance. However, for fastidious bacterial pathogens, CFU inevitably underestimates the threat for the consumer. For viruses, for which viablility detection is often lacking, alternative detection via PCR methods are increasingly accepted within a legislative context (EFSA, 2012).

Conflict of interest

This manuscript was prepared as one of the duties of the German National Reference Laboratory for *Campylobacter*. The authors declare no conflict of interest, which could influence the content or opinions presented in the manuscript.

References

Ahmed MF, Schulz J, Hartung J (2013): Survival of *Campylobacter jejuni* in naturally and artificially contaminated laying hen feces. Poult Sci 92: 364–369.

Amann RI, Ludwig W, Schleifer KH (1995): Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59: 143–169.

Baumgartner A, Felleisen R (2011): Market surveillance for contamination with thermotolerant *Campylobacter*s on various categories of chicken meat in Switzerland. J Food Prot 74: 2048–2054.

Berghaus RD, Thayer SG, Law BF, Mild RM, Hofacre CL, Singer RS (2013): Enumeration of Salmonella and *Campylobacter* spp. in environmental farm samples and processing plant carcass rinses from commercial broiler chicken flocks. Appl Environ Microbiol 79: 4106–4114.

Boulos L, Prevost M, Barbeau B, Coallier J, Desjardins R (1999): LIVE/DEAD BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J Microbiol Methods 37: 77–86.

Bui XT, Wolff A, Madsen M, Bang DD (2012): Reverse transcriptase real-time PCR for detection and quantification of viable *Campylobacter jejuni* directly from poultry faecal samples. Res Microbiol 163: 64–72.

Callicott KA, Hargardottir H, Georgsson F, Reiersen J, Frigriksdottir V, Gunnarsson E, Michel P, Bisaillon JR, Kristinsson KG, Briem H, Hiett KL, Needleman DS, Stern NJ (2008): Broiler *Campylobacter* contamination and human *campylobacteriosis* in Iceland. Appl Environ Microbiol 74: 6483–6494.

Contreras PJ, Urrutia H, Sossa K, Nocker A (2011): Effect of PCR amplicon length on suppressing signals from membrane-compromised cells by propidium monoazide treatment. J Microbiol Methods 87: 89–95.

Cox NA, Berrang ME, Stern NJ, Musgrove MT (2001): Difficulty in recovering inoculated *Campylobacter jejuni* from dry poultry-associated samples. J Food Prot 64: 252–254.

Davey HM (2011): Life, death, and in-between: meanings and methods in microbiology. Appl Environ Microbiol 77: 5571–5576.

EFSA (2010a): Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses in the EU, 2008; Part A: *Campylobacter* and *Salmonella* prevalence estimates. EFSA J 8: 1503.

EFSA (2010b): Analysis of the baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses, in the EU, 2008; Part B: Analysis of factors associated with *Campylobacter* colonisation of broiler batches and with *Campylobacter* contamination of broiler carcasses; and investigation of the culture method diagnostic characteristics used to analyse broiler carcass samples. EFSA J 8: 1522.

EFSA (2011): Scientific Opinion on *Campylobacter* in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain. EFSA J 9: 2105.

EFSA (2012): Scientific opinion on Norovirus (NoV) in oysters: methods, limits and control options. EFSA J 10: 2500.

EFSA, ECDC (2014): The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. EFSA J 12: 3547.

Ferullo DJ, Cooper DL, Moore HR, Lovett ST (2009): Cell cycle synchronization of *Escherichia coli* using the stringent response, with fluorescence labeling assays for DNA content and replication. Methods 48: 8–13.

Fittipaldi M, Nocker A, Codony F (2012): Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. J Microbiol Methods 91: 276–289.

Flekna G, Stefanic P, Wagner M, Smulders FJ, Mozina SS, Hein I (2007): Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. Res Microbiol 158: 405–412.

Flint A, Sun YQ, Butcher J, Stahl M, Huang H, Stintzi A (2014): Phenotypic screening of a targeted mutant library reveals *Campylobacter jejuni* defenses against oxidative stress. Infect Immun 82: 2266–2275.

Garcia AB, Kamara JN, Vigre H, Hoorfar J, Josefsen MH (2013): Direct Quantification of *Campylobacter jejuni* in Chicken Fecal Samples Using Real-Time PCR: Evaluation of Six Rapid DNA Extraction Methods. Food Anal Method 6: 1728–1738.

Giao MS, Wilks SA, Azevedo NF, Vieira MJ, Keevil CW (2009): Validation of SYTO 9/propidium iodide uptake for rapid detection of viable but noncultivable Legionella pneumophila. Microb Ecol 58: 56–62.

Habib I, Sampers I, Uyttendaele M, Berkvens D, De Zutter L (2008): Baseline data from a Belgium-wide survey of *Campylobacter* species contamination in chicken meat preparations and considerations for a reliable monitoring program. Appl Environ Microbiol 74: 5483–5489.

Hammes F, Berney M, Egli T (2011): Cultivation-independent assessment of bacterial viability. Adv Biochem Eng Biotechnol 124: 123–150.

Hazeleger WC, Wouters JA, Rombouts FM, Abee T (1998): Physiological activity of *Campylobacter jejuni* far below the minimal growth temperature. Appl Environ Microbiol 64: 3917–3922.

He Y, Chen CY (2010): Quantitative analysis of viable, stressed and dead cells of *Campylobacter jejuni* strain 81-176. Food Microbiol 27: 439–446.

ISO 10272-2 (2006): ISO/TS 10272-2: Microbiology of food and animal feeding stuffs – Horizontal method for detection and enumeration of *Campylobacter* spp. – Part 2: Colony-count technique. Geneva, Switzerland.

Janzer VA, Buhler C, Krüger NJ, Stingl K, (2014): Real-time PCR detection of *Campylobacter* in chicken meat and faeces. In: Tagungsband DVG-Fachgruppe "Bakteriologie und Mykologie", Freising. Verlag der DVG Service GmbH, Gießen, pp. 172–173.

Josefsen MH, Jacobsen NR, Hoorfar J (2004): Enrichment followed by quantitative PCR both for rapid detection and as a tool for quantitative risk assessment of food-borne thermotolerant *campylobacter*s. Appl Environ Microbiol 70: 3588–3592.

Josefsen MH, Lofstrom C, Hansen TB, Christensen LS, Olsen JE, Hoorfar J (2010): Rapid quantification of viable *Campylobacter* bacteria on chicken carcasses, using real-time PCR and propidium monoazide treatment, as a tool for quantitative risk assessment. Appl Environ Microbiol 76: 5097–5104.

Keer JT, Birch L (2003): Molecular methods for the assessment of bacterial viability. J Microbiol Methods 53: 175–183.

Krüger N-J, Buhler C, Iwobi AN, Huber I, Ellerbroek L, Appel B, Stingl K (2014): "Limits of control" – crucial parameters for a reliable quantification of viable *Campylobacter* by real-time PCR. PLOS One 9: e88108.

LFGB (German Food and Feed Code) (2013): Detection of *Campylobacter* spp. in minced meat – real-time PCR methods, § 64, L-06.32-1, Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), Beuth GmbH, Germany.

Loozen G, Boon N, Pauwels M, Quirynen M, Teughels W (2011): Live/dead real-time polymerase chain reaction to assess new therapies against dental plaque-related pathologies. Mol Oral Microbiol 26: 253–261.

Lübeck PS, Wolffs P, On SL, Ahrens P, Radstrom P, Hoorfar J (2003): Toward an international standard for PCR-based detection of food-borne thermotolerant *Campylobacter*s: assay development and analytical validation. Appl Environ Microbiol 69: 5664–5669.

Matsuoka H, Nakano K, Takatani N, Yoshida T, Igimi S, Saito M (2014): Flow cytometric method for in situ preparation of standard materials of a small defined number of microbial cells with colony-forming potentiality. J AOAC Int 97: 479–483.

Musgrove MT, Berrang ME, Byrd JA, Stern NJ, Cox NA (2001): Detection of *Campylobacter* spp. in ceca and crops with and without enrichment. Poult Sci 80: 825–828.

Nebe-von-Caron G, Stephens PJ, Hewitt CJ, Powell JR, Badley RA (2000): Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. J Microbiol Methods 42: 97–114.

Nkuipou-Kenfack E, Engel H, Fakih S, Nocker A (2013): Improving efficiency of viability-PCR for selective detection of live cells. J Microbiol Methods 93: 20–24.

Nocker A, Cheung CY, Camper AK (2006): Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods 67: 310–320.

Pacholewicz E, Swart A, Lipman LJ, Wagenaar JA, Havelaar AH, Duim B (2013): Propidium monoazide does not fully inhibit the detection of dead *Campylobacter* on broiler chicken carcasses by qPCR. J Microbiol Methods 95: 32–38.

Reich F, Atanassova V, Haunhorst E, Klein G (2008): The effects of *Campylobacter* numbers in caeca on the contamination of broiler carcasses with *Campylobacter*. Int J Food Microbiol 127: 116–120.

Rodgers JD, Lawes JR, Vidal AB, Ellis-Iversen J, Ridley A, Pleydell EJ, Powell LF, Toszeghy M, Stapleton K, Clifton-Hadley FA (2012): Characteristics and comparative performance of direct culture, direct PCR and enumeration methods for detection and quantification of *Campylobacter* spp. in broiler caeca. Vet Microbiol 159: 390–396.

Rosenquist H, Sommer HM, Nielsen NL, Christensen BB (2006): The effect of slaughter operations on the contamination of chicken carcasses with thermotolerant *Campylobacter*. Int J Food Microbiol 108: 226–232.

Rudi K, Moen B, Dromtorp SM, Holck AL (2005): Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl Environ Microbiol 71: 1018–1024.

Schaechter M, Maaloe O, Kjeldgaard NO (1958): Dependency on medium and temperature of cell size and chemical composition during balanced grown of *Salmonella* Typhimurium. J Gen Microbiol 19: 592–606.

Seinige D, Krischek C, Klein G, Kehrenberg C (2014): Comparative analysis and limitations of ethidium monoazide and propidium monoazide treatments for the differentiation of viable and nonviable *Campylobacter* cells. Appl Environ Microbiol 80: 2186–2192.

Stern NJ, Reiersen J, Lowman R, Bisaillon JR, Fridriksdottir V, Gunnarsson E, Hiett KL, Campy-on-Ice Consortium (2005): Occurrence of *Campylobacter* spp. in cecal contents among commercial broilers in iceland. Foodborne Pathog Dis 2: 82–89.

Stern NJ, Robach MC (2003): Enumeration of *Campylobacter* spp. in broiler feces and in corresponding processed carcasses. J Food Prot 66: 1557–1563.

Stingl K, Knüver MT, Vogt P, Buhler C, Krüger NJ, Alt K, Tenhagen B-A, Hartung M, Schroeter A, Ellerbroek L, Appel B, Käsbohrer A (2012): Quo vadis? – Monitoring *Campylobacter* in Germany. Eur J Microbiol Immunol 2: 88–96.

Sträuber H, Müller S (2010): Viability states of bacteria – specific mechanisms of selected probes. Cytometry. Part A. J Int Soc Anal Cytol 77: 623–634.

Tholozan JL, Cappelier JM, Tissier JP, Delattre G, Federighi M (1999): Physiological characterization of viable-but-nonculturable *Campylobacter jejuni* cells. Appl Environ Microbiol 65: 1110–1116.

Van Gerwe TJ, Bouma A, Jacobs-Reitsma WF, van den Broek J, Klinkenberg D, Stegeman JA, Heesterbeek JA (2005): Quantifying transmission of *Campylobacter* spp. among broilers. Appl Environ Microbiol 71: 5765–5770.

Yamasaki M, Igimi S, Katayama Y, Yamamoto S, Amano F (2004): Identification of an oxidative stress-sensitive protein from *Campylobacter jejuni,* homologous to rubredoxin oxidoreductase/rubrerythrin. FEMS Microbiol Lett 235: 57–63.

Yanez MA, Nocker A, Soria-Soria E, Murtula R, Martinez L, Catalan V (2011): Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR. J Microbiol Methods 85: 124–130.

Address for correspondence:
Dr. Kerstin Stingl
Bundesinstitut für Risikobewertung
Abteilung Biologische Sicherheit
Nationales Referenzlabor für *Campylobacter*
12277 Berlin
Germany
kerstin.stingl@bfr.bund.de