Preparation of quantitative thermophilic *Campylobacter* spp. reference material for use in proficiency tests or ring trials for method validation

Laboratory Protocol of the National Reference Laboratory for *Campylobacter*,
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The National Reference Laboratory (NRL) for *Campylobacter* is located at the German Federal Institute for Risk Assessment (BfR). The work of the NRL focuses on routine and research work on the characterisation and differentiation of *Campylobacter* isolates originating from animals, food and environmental samples. Various molecular biological methods, including whole genome sequencing, are available for epidemiological investigations. The NRL organizes interlaboratory proficiency tests for qualitative and quantitative detection of *Campylobacter* in relevant matrices, e.g. chicken meat/skin, raw milk and chicken caecal content and produces quantitative reference standards such as the following laboratory protocol for the preparation of quantitative thermophilic *Campylobacter* spp. reference material for use in proficiency tests or ring trials for method validation.

1 Background

Quantitative reference material is needed for several applications, such as performance testing of culture media, quality control for food business operators and competent authorities and for quantitative proficiency tests. Here, we provide a protocol for the production and for quality check of quantitative thermophilic *Campylobacter* reference material (*C. jejuni and C. coli*). *Campylobacter* is a fastidious bacterium requiring complex nutrients and microaerobic atmosphere to grow in vitro. One of the crucial points of the protocol is to optimize culture conditions and to adequately maintain the bacterium's colony formig unit (CFU) capacity (see overview in Fig. 1).

2 Strains and growth conditions

C. jejuni NCTC 11168 or DSM 4688 and *C. coli* WDCM 00004 were cultured from -80°C cryobank stocks on Columbia blood agar supplemented with 5% defibrinated sheep blood in a microaerobic incubator with 5% O_2, 10% CO_2 and 85% N_2 at 37 °C for 24±2h. When liquid cultures were used the microaerobic atmosphere was established in anaerobic jars, which were twice evacuated to -0.7 bar and refilled with a gas mixture of 5% O_2, 10% CO_2 and 85% N_2. As quality control, re-growth from a single cryobank bead to a clearly visible amount of cell material was feasible during 24±2h at 37°C on ColbA.

3 Growth in liquid culture and cryo-conservation

Campylobacter strains grown for 24±2h from -80 °C stocks on ColbA were subcultured for another for 18±2 h under the same conditions. Subsequently, the cells were inoculated in sterile-filtered prewarmed brain heart infusion broth to an initial OD_{600} ~ 0.3. The bacteria were incubated in prewarmed anaerobic jars, which were twice evacuated and refilled with microaerobic atmosphere at 140 rpm and 37°C until stationary phase for approximately 5-9h (OD_{600} ~ 1.5-2). The OD_{600} was determined and based on an assumed generation time of 1.3h for *C. coli* and 1.6h for *C. jejuni*, the initial inoculation time was calculated and the appropriate inoculum and a 10-fold diluted incoculum was added to a prewarmed BHI medium for growth over night (~15-18h) to reach an early stationary phase of OD_{600} between 0.8 and 1.2. The OD_{600}, motility and homogeneous morphology were checked and, if inappropriate (i.

e. the bacteria were heterogeneous in length and hardly motile), the culture was again diluted to OD_{600} ~0.3 for growth over day and after subsequent dilution incubated again over night.

Cryomedium was composed of Bolton basis without selective supplements, but with 5% laked horse blood and 10% cell culture tested glycerol and was prepared fresh and cooled on ice. After choosing one of the cultures with an appropriate quality, the cells were added at a distinct concentration to ice cold cryomedium assuming a maximal reduction of 0.7 $\log_{10}$ CFU per ml due to freezing. We considered an OD_{600} of 0.2 to correspond to 8.5 $\log_{10}$ CFU/ml before freezing. Cryo vials were filled with 0.1 to 1 ml aliquots of the cryoculture under constant gentle stirring on ice. Ten vials (e. g. every 15th vial for total 150 filled vials) were numbered according to their position in the filling line in order to cover the whole filling process and later used for the homogeneity test. The cryoculture, including the filled cryo vials, were kept on ice for at least 2h but not longer than 4h, starting with the timepoint of inoculation of the cryomedium. Subsequently, the vials were shock-frozen in liquid nitrogen for at least ten seconds before transfer to dry ice and final transfer to pre-cooled boxes for long-term storage at -80°C. Temperature variations during filling and, subsequently, during storage should be avoided in order to maintain CFU capacity.

4 Analysis of performance criteria

Enumeration was performed from the culture before freezing. After freezing and storage for at least one day, enumeration was performed for two vials in replicate and two dilutions in order to estimate the loss of CFU due to the freeze-thaw process and to exactly determine one appropriate dilution for performing the homogeneity test from ten vials taken along the filling process. For this purpose, vials were thawed for 15 to 30 min at room temperature (depending on the volume) and, thereafter, placed on ice for at least 30 min up to 3 hours in order to guarantee optimal CFU recovery. Subsequently, bacterial suspensions were diluted in buffered 1% peptone water and plated on two ColbA plates for microaerobic incubation for 48 h at 41.5°C.

4.1 Homogeneity assessment

Ten vials per batch were chosen along the whole filling line and tested for homogeneity in compliance with ISO 13528:2015. From each vial three test portions (replicates) were examined by enumerating one appropriate dilution on two ColbA plates per replicate and vial. The appropriate dilution was calculated on the basis of the CFU obtained after freezing (see above), intending to obtain ~50 colonies per plate. For this purpose, the dilution was performed in buffered 1% peptone for vial 1 to 10 and 100µl of diluted suspension was plated on two ColbA plates (replicate 1). Thereafter, the dilution series was withdrawn and vial 1 to 10 was again diluted and plated in duplicate (replicate 2). In analogy, replicate 3 was performed for the ten vials. The plates were incubated for 48h at 41.5°C under microaerobic conditions. The between-sample standard deviation (s_s) was compared with the standard deviation for proficiency assessment σ_{pt}. According to ISO 13528:2015, homogeneity is considered sufficient if s_s does not exceed 0.3-times the standard deviation for proficiency assessment σ_{pt}. σ_{pt} defines the acceptable level of variation between laboratories for a particular test. In case no historical data for the reference material are available, σ_{pt} is set to a fixed value of 0.35 $\log_{10}$ for microbial proficiency testing schemes according to ISO 22117:2018. Since a technical high intra-standard deviation might mask non-homogeneous material, we also defined a maximal difference between all measured $\log_{10}$ CFU values of 0.5 $\log_{10}$ and a maximal intra-standard deviation of 0.1 $\log_{10}$.

4.2 Stability assessment

For stability assessment, at least five time points were chosen, at which two vials per batch were enumerated according to ISO 13528:2015. The two replicates were prepared and plated as described above. As first and second stability time point, data of the two vials after direct freezing and two vials randomly chosen from the homogeneity test dataset were used. The reference material was considered stable if the absolute difference between the overall average of the homogeneity test ($\bar{x}$) and the overall average of the stability test ($\bar{y}$) results did not exceed 0.3-times the standard deviation for proficiency assessment σ_{pt} (defined as 0.35 $\log_{10}$, see above; ISO 13528:2015) plus expanded uncertainty. Hence we checked whether ($|\bar{x} - \bar{y}|$) < 0.3 σ_{pt} + 2u (ISO 13528:2015, B.5.2), with u = $s_{IR}/\sqrt{n}$ and n, number of stability time points. The uncertainty was estimated from intralaboratory standard deviation (s_{IR}) following ISO 19036:2020, 5.2.2.3.2.

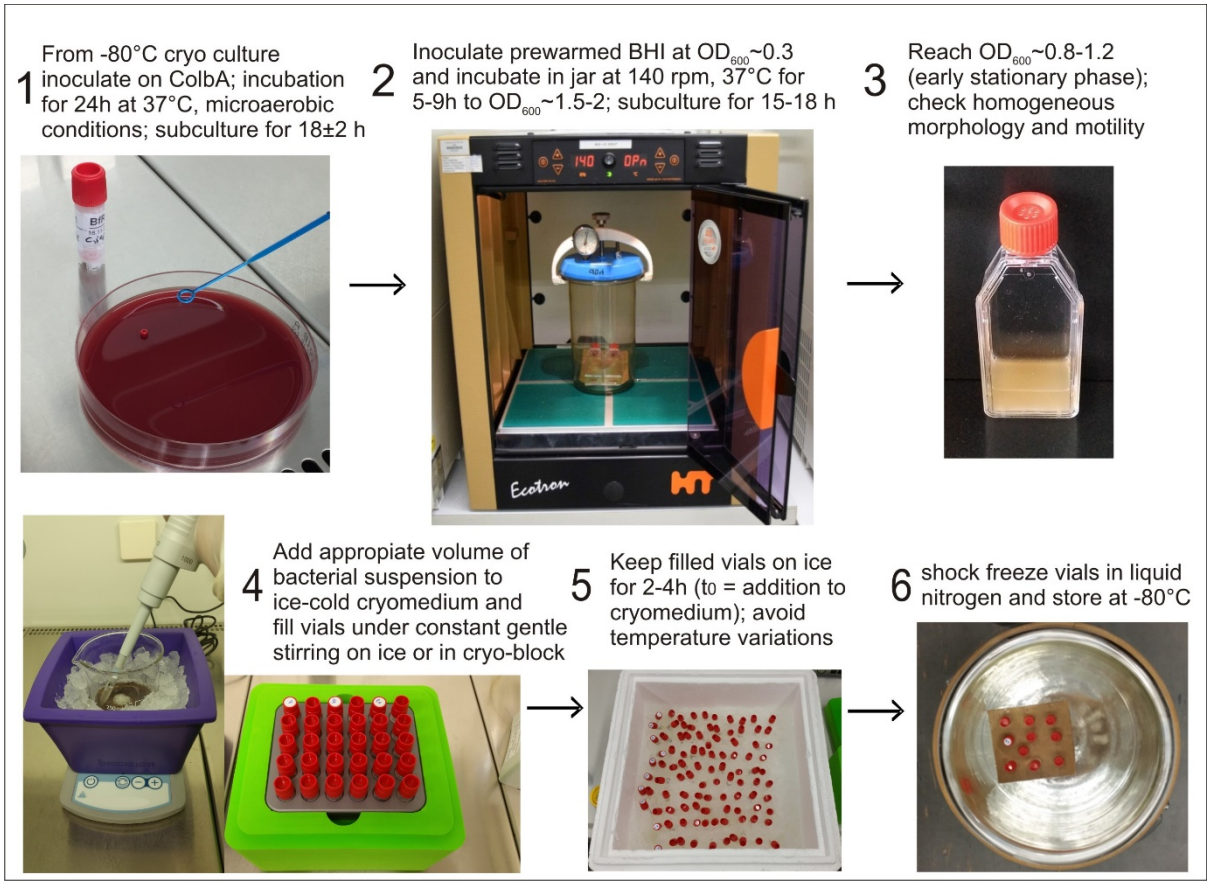

Figure 1: Overview of the procedure for production of quantitative reference material of thermophilic *Campylobacter* spp.

5 References

ISO 13528:2015. Statistical methods for use in proficiency testing by interlaboratory comparison. International Standard Organization, Geneva, Switzerland.

ISO 22117:2018. Microbiology of food and animal feeding stuffs - Specific requirements and guidance for proficiency testing by interlaboratory comparison. International Organization for Standardization, Geneva, Switzerland.

ISO 19036:2020. Microbiology of the food chain – Estimation of measurement uncertainty for quantitative determinations. International Organization for Standardization, Geneva, Switzerland.

About the BfR

The German Federal Institute for Risk Assessment (BfR) is a scientifically independent institution within the portfolio of the Federal Ministry of Food and Agriculture (BMEL) in Germany. The BfR advises the Federal Government and the States (‘Laender’) on questions of food, chemical and product safety. The BfR conducts its own research on topics that are closely linked to its assessment tasks.