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Double staining (CTC-DAPI) for detection and enumeration of viable but non-culturable Campylobacter jejuni cells

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Summary – Direct microscopic enumeration of viable Campylobacter jejuni cells (ie, respiring bacteria) were performed in both culturable and non-culturable states. Five different C. jejuni strains were used, including a reference strain, ATCC 33291. Cells from all five strains were incubated alone with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), a redox dye. It was reduced by an electron transport chain to an insoluble red fluorescent CTC formazan salt, which accumulated intracellularly. The presence of these red CTC crystals in the bacteria cells was indicative of cellular respiratory activity. Counterstaining with 4'-6-diamino-2-phenylindole (DAPI), which fluoresces in blue, made a suitable contrast and allowed simultaneous enumeration of total and viable bacteria on a single filter. Four hours of incubation with 5 mM CTC under a microaerobic atmosphere was found to be the optimal condition yielding the maximum number of respiring cells (both culturable and non-culturable). When used in combination with standard culture techniques, double staining makes it possible to monitor the viable but non-culturable cells of C. jejuni obtained by starvation more easily than with the direct viable count procedure.

Campylobacter jejuni / double staining / starvation / viable but non-culturable

Résumé – Double coloration (CTC-DAPI) pour la détection et le comptage des cellules viables mais non cultivables de Campylobacter jejuni. Des dénombrements microscopiques des cellules viables (ie présentant une activité respiratoire) ont été réalisés sur des cellules de C. jejuni à l’état cultivable et à l’état non cultivable. Cinq souches différentes de C. jejuni ont été utilisées, dont une souche de référence, ATCC 33291. Les cellules de chacune des souches ont été incubées en pré-

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sence de 5-cyano-2,3-ditolyl tétrazolium chloride (CTC), colorant redox. Celui-ci a été réduit par les électrons de la chaîne respiratoire en CTC formazan, précipité rouge fluorescent, qui s’est accumulé dans la cellule. La présence de ces pigments rouges fluorescents intracellulaires témoignait d’une activité respiratoire. Une contre coloration avec le 4’-6 diamino 2 phényl indole (DAPI), qui fluoresce en bleu, a permis d’effectuer sur une même préparation un dénombrement bactérien total et un dénombrement des bactéries viables. Les conditions optimales permettant de détecter le maximum de cellules viables, à l’intérieur d’une population de cellules non cultivables, ont correspondu à 4 h d’incubation en microaérobiose, avec une concentration en CTC de 5 mM. Couplée à une technique classique de culture, cette double coloration permet de suivre les cellules viables non cultivables de *C jejuni* obtenues par carence en nutriments, plus facilement que par le dénombrement direct des cellules viables.

*Campylobacter jejuni / double coloration / carence en nutriments / viable non cultivable*

**INTRODUCTION**

A viable but non-culturable (VNC) bacterial state was originally detected in studies in environmental microbiology (Roszak and Colwell, 1987) and then demonstrated for a number of human pathogens: *Escherichia coli* (Xu et al, 1982), *Salmonella enteritidis* (Roszak et al, 1984), *Vibrio cholerae* (Colwell et al, 1985), *Legionella pneumophila* (Hussong et al, 1987) and *Campylobacter jejuni* (Rollins and Colwell, 1986). The presence of VNC cells poses a major public health problem since they cannot be detected by traditional culturing methods and the cells remain potentially pathogenic under favorable conditions (Stern et al, 1994; Rahman et al, 1994; Ravel et al, 1995).

The detection and study of VNC cells requires non-culturing techniques in order to detect the bacterial cell viability. In most studies, this has been assessed by the Kogure procedure based on cellular elongation in the presence of DNA gyrase inhibitors (Kogure et al, 1979). Another means of distinguishing metabolically active from inactive cells consists in using tetrazolium salts, which are redox dyes that compete with molecular oxygen as an artificial electron acceptor and which are converted to insoluble formazan crystals that accumulate in active cells. Among these dyes, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5 phenyltetrazolium chloride (INT) was the first to be used with bacteria cells. However, its reduced formazan form is not fluorescent and requires visualization by bright-field microscopy (Zimmerman et al, 1978). Another dye, 5-cyano-2,3-ditolyl tetrzolium chloride (CTC), has also been used since it is transformed into a fluorescent red precipitate readily visualized by epifluorescence microscopy (Severin et al, 1985). Because of the fluorescent nature of its reduced formazan form, CTC has proven superior to INT for viable cell (ie, respiring cells) counts (Rodriquez et al, 1992).

*C jejuni* is currently recognized as a leading foodborne pathogen (Skirrow, 1990; Notermans and Hoogenboom-Verdegaal, 1992). During the last 10 years, a marked increase in the incidence of human enteric campylobacteriosis has been reported in many countries (Taylor, 1992). Two morphological forms have generally been described for *C jejuni*: culturable spiral forms and degenerating coccoid forms. The transition from spiral to coccoid forms (Boucher et al, 1994) depends on many factors (strains, temperature, pH, osmolarity, medium, etc) and requires variable time intervals. During transition, viable non-culturable spiral and coccoid forms of *C jejuni* exist (Rollins and Colwell, 1986; Beumer et al, 1992; Medema et al, 1992). It is controversial however as to whether a transition from non-culturable to culturable forms occurs. This possibility is crucial because
of the potential infectiousness of the former (Jones et al, 1991). The present study describes a double-staining technique using CTC and 4’-6 diamino-2 phenylindole (DAPI) fluorochromes to detect and count the total and VNC C. jejuni simultaneously. Appropriate incubation times and CTC concentrations were considered.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions**

We used five different C. jejuni strains, namely Bf, 79, 85, C-1 and ATCC 33291, all of which had confirmed hippurate hydrolysis, nalidixic acid sensitivity and cephalotin resistance. For each test, the strains were grown on Columbia agar (Unipath, Basingstoke, UK) supplemented with 5% lysed horse blood for 24 h at 42 °C under a microaerophilic atmosphere (BBL Campypack, Cockeysville, USA). Two subcultures were performed under similar conditions before the inocula were starved. The inoculum consisted of about 10⁸ bacteria/mL.

**Starvation**

Two methods were used to obtain non-culturable cells (starved cells) of C. jejuni. For the microcosm water method described by Rollins and Colwell (1986), the incubated cells were suspended in bottles containing 500 mL of filtered (Nalgene-0.2 μm, Rochester, USA), sterilized surface water adjusted to pH 6 ± 0.1 (NaOH 0.1 N). The bottles were then shaken (100 rpm) at +4 °C. The second system involved suspending the cells in phosphate-buffered saline (PBS) (pH 7.2 ± 0.1) at +4 °C without shaking.

**Spread plate counts**

Culturability was assayed by spread plate counts. Serially diluted samples in peptone water were spread in triplicate onto 5% lysed horse blood Columbia agar. After 48-h incubation at 42 °C under a microaerophilic atmosphere, colony-forming units (CFUs) at appropriate dilutions were counted and referred to the original sample.

**CTC-DAPI staining.**

At corresponding times, samples from the C. jejuni suspensions were removed and the cells were stained with CTC and DAPI, according to a modified technique of Rodriguez et al (1992). Half a milliliter (0.5 mL) of brain–heart infusion (Biokar, Beauvais, France) and 100 μL of a 0.05 g/L solution of pyruvic acid (Sigma, St Louis, USA) were added to 0.5 mL of the bacterial suspension to be analyzed in order to stimulate cellular respiration. CTC (Polysciences, Warringon, USA) was diluted to obtain a final concentration of 5 mM, and the mixture was then incubated for times ranging from 0.5 to 4 h at 37 °C under a microaerophilic atmosphere. Cells were subsequently harvested by filtration through an isopore polycarbonate black membrane filter (0.2-μm pore size, 25 mm in diameter; Millipore, Ireland) and covered with a 5 μg/mL DAPI solution for 5 min for counterstaining purposes. Finally, the stain was removed by filtration, and the filter was air-dried and mounted in non-fluorescent immersion oil (Olympus, Japan) before a coverslip was added. An Olympus BX40 epifluorescent microscope equipped with a BH2-RFL-T3 100-W light source was used to examine the filters at a magnification of 1000. Observations were performed with a fluorescence light fitted with a 405-nm excitation filter and a 455-nm dichroic mirror, allowing simultaneous visualization of both dyes. Counting was carried out randomly on the basis of 20 microscopic fields per filter. For each sample, four filters were counted. Respiring cell counts (RCCs), showing CTC formazan crystals, and total cell counts (TCCs), with staining by DAPI (ie, viable and non-viable), were determined. Results were expressed as the number of corresponding bacteria per milliliter of the original sample, and percentages of RCCs relative to TCCs were determined. Experiments were conducted in triplicate.

**Direct viable counts procedure**

In order to compare respiring cell counts to direct viable counts, a modified technique of Kogure et al (1979) was used. At corresponding times,
1 mL of the cell suspensions were resuspended in 9 mL BHI (Biokar, Beauvais, France) to which yeast extract (Difco, Detroit, USA) (0.025 mg/mL) and nalidixic acid (Sigma, St Louis, USA) (10 µg/mL) were added. The mixture was incubated for 24 h at 42 °C under a microaerophilic atmosphere. Cells were subsequently harvested by filtration through an isopore polycarbonate black membrane filter (0.2 µm pore size, 25 mm diameter; Millipore, Ireland) and covered with a 0.025% acridine orange solution for 5 min. The filters were prepared and examined as described above.

Statistical analysis

Statistical tests were performed with the StatView Program (Apple computer). All counts reported are the averages of at least three determinations, and the coefficients of variation between replicate experiments were less than 12%. Differences between averages were calculated by one-way analysis of variance. Probabilities less than or equal to 0.05 were considered significant. Bars show the maximal population variance observed.

RESULTS

CTC incubation time

The CTC assay was performed with both culturable and non-culturable (starved) cells from different C jejuni strains. Figures 1 and 2 show the proportion of respiring bacteria according to CTC incubation time. The peaks of CTC reduction were reached after 4 h of incubation for both culturable and starved cells from C jejuni strains Bf, 79 and 85. However, among the culturable cells, the numbers of respiring cells detected at 1 h of CTC incubation were already greater than 90% of those obtained at 4 h (maximum number), whereas among the starved cells this percentage was only about 56% for the same length of time.

In order to appreciate the efficiency of CTC reduction in Campylobacter, further investigations were carried out with other C jejuni strains: a recent human isolate designated as the C-1 strain for culturable cells, and the ATCC 33291 strain for starved cells (40 days in PBS at 4 °C without shaking). In all cases, the maximum numbers and percentages were detected after 4 h of CTC incubation under a microaerophilic atmosphere (data not shown).

When cells were incubated for longer than 4 h, the respiring cell counts were much more difficult to establish because of the occurrence of non-specific reactions.

CTC reduction by C jejuni cells

Microscopic enumerations of total and respiring cells, together with CFUs, were used to monitor the transition of C jejuni cells to the viable but non-culturable state in microcosm water. Respiration was determined by the CTC assay. Survival was assayed with Bf, 79 and 85 C jejuni strains, and the results are presented in figures 3, 4 and 5, respectively. In each case, the total cell count appeared constant (about 10⁸/mL) over the experimental period. VNC state was reached after 16 days of starvation for strain Bf and after 14 days for strains 79 and 85, as indicated by a decrease to less than one CFU per milliliter. However, at that time, around 10⁶ bacteria per milliliter showed CTC formazan crystals inside cells, indicating the presence of respiring cells (ie, viable but non-culturable cells). Respiring cell counts and direct viable counts were quite similar for all strains tested. Similar counts were detected 2 weeks later (day 30), at the end of the experiment.

DISCUSSION

These studies show that CTC is reduced by C jejuni cells (ie, respiring cells) in both culturable and non-culturable states and that counterstaining with DAPI allowed the enumeration of both the total number of cells and the number of metabolically active cells.
**Fig 1.** Culturable cells of *Campylobacter jejuni* (<20 h culture). Percentage of CTC reducing bacteria according to incubation time: ■, B1; □, 79; ▪, 85.

**Fig 2.** Starved cells of *Campylobacter jejuni* (15 days). Percentage of CTC reducing bacteria according to incubation time. Percentages were calculated with respect to the maximum number of CTC reducing cells (4 h): ■, B1; □, 79; ▪, 85.
Fig 3. Numeration of *Campylobacter jejuni* cells strain Bf: ⋅○⋅, culturable count; ⋅●, direct viable count; ⋅×, respiring cell count; ⋅▲, total cell count.

Fig 4. Numeration of *Campylobacter jejuni* cells strain 79: ⋅○⋅, culturable count; ⋅●, direct viable count; ⋅×, respiring cell count; ⋅▲, total cell count.
on the same filter. Since Rodriguez et al (1992) first demonstrated the use of CTC in studies other than with ascites Erhlich tumor cells (Stellmach and Severin, 1987), this redox dye has been used with several pure strains such as Escherichia coli (Pyle et al, 1995), Pseudomonas putida (Walsh et al, 1995) and Listeria monocytogenes (Bovill et al, 1994), as well as with environmental samples (Smith et al, 1994; Yu et al, 1995) and bacterial biofilms (Huang et al, 1995). However, a specific method for C jejuni cells has not yet been reported. The survival of C jejuni has been demonstrated by the cellular elongation method (DVC) and the INT procedure or CFU determinations (Rollins and Colwell, 1986). Comparative studies of the CTC procedure and INT or DVC methods have been reported (Rodriguez et al, 1992; Coallier et al, 1994), which have clearly shown that the CTC procedure is the technique of choice, mainly because of its fluorescent nature and shorter incubation time. Our previous results for DVC with the Bf, 79 and 85 strains (data not shown) were quite similar to those for the CTC assay, which confirms the validity of both methods for counting and detecting viable C jejuni cells. However, cell elongation for C jejuni cells is a very slow process (more than 7 h) and it is not always easy to detect by microscopic observation because of the transition to coccoid forms under stress factors. In these coccoid forms, DVC is much more difficult to interpret than in bacillus (spiral) forms.

The quantification of respiring bacteria by CTC reduction is subject to experimental conditions, particularly for incubation times and CTC concentrations. Although our results, based on maximum counts of CTC reducing cells, indicate an optimal 4-h incubation period for all five strains used, shorter times (2 h) did not show any significant differences. Longer times (> 4 h) resulted in extracellular deposit formation.
which made counting difficult. The optimal CTC incubation time was originally considered to be 4 h (Stellmach and Severin, 1987) but was lowered (1 h) in subsequent studies (Schaule et al, 1993) in order to reduce the number of cell divisions among the culturable cells. However, CTC incubation time is crucial for time-limited uptake of CTC by viable non-culturable cells. Furthermore, it is important in the case of microaerophilic bacteria to ensure that a microaerobic atmosphere is generated, which can be achieved within different time periods depending on the system used. Finally, other authors (Coallier et al, 1994) have recommended longer incubation times (6–24 h) and lower CTC concentrations in order to avoid toxic effects and CTC impurities or deposits. Physiological differences between bacterial groups can have an influence on the optimal incubation time needed to produce a maximum of CTC reducing cells.

The CTC concentration added to bacterial suspensions also varies with the studies and the experimental conditions used. Most authors used 5 mM CTC (final concentration), as recommended by Rodriguez et al (1992). Nevertheless, Coallier et al (1994) found that 0.5 mM CTC (at 2 h) and 1 mM CTC (at 6 h) were optimal concentrations, whereas 5 mM yielded the lowest number of CTC reducing cells from water samples. Schaule et al (1993) reported an optimal concentration as low as 0.5 mM CTC for 1 h. Our results indicated that 5 mM CTC, instead of 3 mM, was optimal because of the significantly higher number of respiring cells detected both at 2 and 4 h (data not shown). Furthermore, the very small coccoid forms of non-culturable C jejuni were more easily detected and differentiated from the background when the CTC concentration was increased from 3 to 5 mM.

Counterstaining CTC with DAPI allowed simultaneous determinations of total (red and blue fluorescent) bacteria and viable bacteria. In some instances, mainly among spiral cells, it was possible to distinguish specific areas in which CTC crystals were deposited in cells owing to the contrast between CTC and DAPI fluorescence. Other staining agents traditionally used in direct viable counts, such as acridine orange, have emission wavelengths too close to those of reduced CTC formazan, making it very difficult to distinguish between the total and respiring cells (Boucher et al, 1994). Nevertheless, for some authors, as infra-estimation of respiring bacteria can occur in simultaneous determinations, it is advisable to use an additional microscope filter (420 nm excitation and 490 nm barrier) to count the total and viable cells on a single filter.

On the whole, the double-staining technique proposed here, which uses DAPI for total and CTC for viable (ie, actively respiring bacteria) counts of C jejuni cells (both culturable and non-culturable), appears to be both a simple and rapid procedure for studying the VNC forms of this bacterium. Combined with standard culture techniques, double staining allows the monitoring of viable but non-culturable C jejuni cells obtained by starvation.

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