Lack of response of INT-407 cells to the presence of non-culturuble *Campylobacter jejuni*

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SUMMARY

Many contradictory articles on the infectivity of non-culturuble *Campylobacter jejuni* can be found. We studied the effect of non-culturuble *C. jejuni* in an *in vitro* assay. To prevent the potential effect of a few culturable bacteria in the non-culturuble suspension, INT-407 cells, which mimic the outer cell layer in the small intestines, were exposed to culturable *C. jejuni* suspensions with or without non-culturuble *C. jejuni*. The number of bacteria adhering to and/or invading INT-407 cells and the IL-8 secretion were measured. No differences were found between bacterial suspensions with or without non-culturuble *C. jejuni* added. These findings show that non-culturuble *C. jejuni* do not adhere to or invade INT-407 cells and do not induce an immune response. As previous studies showed a correlation between the used *in vitro* assays and the effect *in vivo*, our study strongly suggests that culturability is a good indicator of the risk for *C. jejuni* infection.

INTRODUCTION

*Campylobacter jejuni* is the leading cause of bacterial foodborne gastroenteritis throughout the world [1–4]. It is a Gram-negative, motile microorganism, which is primarily micro-aerophilic. Remarkably, this pathogen grows within a short temperature range, being unable to multiply at temperatures above 45 °C or below 30 °C. At conditions where *C. jejuni* cannot grow, such as low temperature or in spent medium, it loses its culturability. It is crucial to know whether non-culturuble *C. jejuni* can cause an infection, as in practice the exposure is often measured as the number of culturable *C. jejuni* in a product.

In the literature many contradictory articles on the infectivity of non-culturuble *C. jejuni* can be found. Some authors showed that non-culturuble *C. jejuni* were not infective in chicks, mice and human volunteers [5–8] while others demonstrated that non-culturuble *C. jejuni* were colonizing chicks and mice [9–11]. The inconsistency in the literature about the infectivity of non-culturuble *C. jejuni* might be the result of differences in methods, conditions and strains used. A marked difference between studies is the temperature at which non-culturuble *C. jejuni* were formed. While some authors did not find any infectivity of non-culturuble *C. jejuni* formed at 4 °C [6–8], in all studies reporting on non-culturuble *C. jejuni* causing infection, the non-culturuble *C. jejuni* were formed at 4 °C [9–11], whereas in all experiments using higher temperatures no infectivity was found [5, 8]. Interestingly Hazeleger *et al.* [12] showed that
non-culturable *C. jejuni* formed at 4 °C showed characteristics, including intracellular/extracellular ATP ratio and membrane fatty-acid composition, comparable to culturable *C. jejuni*, whereas non-culturable *C. jejuni* formed at 25 °C were clearly different. The aim of this study was to further elucidate if non-culturable *C. jejuni* formed at 4 °C can be infective.

The non-culturability of suspensions, in studies in which non-culturable *C. jejuni* were found to be infective, is often discussed. If only one or a few undetected culturable bacteria remain present, the observed infectivity might be caused by these undetected culturable bacteria instead of by the non-culturable bacteria. To avoid the misleading effect of a few culturable bacteria, in our study the infectivity of culturable *C. jejuni* suspensions and the infectivity of the same culturable *C. jejuni* suspensions supplemented with a high number of non-culturable *C. jejuni*, formed at 4 °C, were measured. The comparison was made for different doses of culturable *C. jejuni* in the absence and presence of non-culturable *C. jejuni*, as the infectivity might be dose-dependent [13–15].

For our study, INT-407 cells were chosen, based on various studies showing that the human cell lines Caco-2 and INT-407 mimic best the outer cell layer in the small intestines [16, 17]. In preliminary work (data not shown) adhesion and invasion were measurable in both Caco-2 and INT-407 cells, but IL-8 secretion after exposure with *C. jejuni* was only detected in INT-407 cells. The INT-407 cells were used for two *in vitro* infectivity tests. First, the adhesion and invasion assay, which is based on the binding to and entry in host cells of *C. jejuni*, an important factor in the pathogenesis of *C. jejuni* [18]. Second, the IL-8 assay in which the *C. jejuni*-stimulated secretion of the cytokine IL-8, an early signal for the mucosal inflammatory response [19, 20], is determined as a measure for the immune response. A disadvantage of the adhesion and invasion assay is that the outcome is measured by plate counting. If non-culturable *C. jejuni* do not recover their culturability during the assay, but do adhere or invade, the infectivity of non-culturable *C. jejuni* is underestimated. As the IL-8 assay is based on measuring an immune response, even if non-culturable *C. jejuni* do not recover, their effect on the infectivity is likely to be measured by the IL-8 assay. As *in vitro* studies have established that the invasive and adhesive ability of *C. jejuni* strains differ [21–27] and, furthermore, that the *C. jejuni*-stimulated IL-8 secretion is strain dependent [28, 29] four *C. jejuni* strains were selected. *C. jejuni* 70.2 and BF were chosen as for these strains the formation of infective non-culturable *C. jejuni* has been described by Cappelier et al. [9, 30]. C356 and 82/69 were selected for their good adherence and invasion properties found in preliminary research (data not shown).

**METHODS**

**Culturing *C. jejuni* strains**

Strains were stored at −70 °C in brain heart infusion broth (BHI, Difco, Sparks, MD, USA) plus 30% (v/v) glycerol in cryovials. For culturing *C. jejuni* strains 70.2 and BF (INRA, Nantes, France; both isolated from human faeces), C356 (ID-Lelystad, The Netherlands; isolated from chicken faeces) and 82/69 (ID-Lelystad; isolated from chicken faeces, same serotype also found in human faeces), the content of one vial (0.5 ml) was thawed and put in a wide-necked Erlemeyer flask with 50 ml BHI. The flask was incubated while shaking at 100 rpm in a custom-made incubator (NuAire, Plymouth, MN, USA) with a micro-aerobic atmosphere (10% O₂, 5% CO₂, 85% N₂) at 37 °C. After ±24 h, 0.5 ml was subcultured in 100 ml fresh BHI and incubated under the same conditions for ±16 h. These suspensions were used in the cell line assays.

**Culturability**

Plate counts were performed by spread plating 0–1 ml of appropriate decimal dilutions of bacterial suspensions in sterile peptone (Difco, 1 g/l) saline (Merck, Amsterdam, The Netherlands; 9 g/l NaCl) solution on Columbia agar base with 5% (v/v) defibrinated horse blood (CAB, Oxoid, Basingstoke, UK). The plates were incubated micro-aerobically at 37 °C in a jar with BBL® Campypak (Becton Dickinson, Sparks, MD, USA) for 72 h.

**Non-culturable *C. jejuni* suspensions**

To obtain non-culturable *C. jejuni*, strains were cultured as described above. After culturing, the bacterial suspensions (±10⁹ *C. jejuni*/ml) were stored aerobically without shaking at 4 °C in wide-necked Erlemeyer flasks covered with cottonwool and kitchen foil to prevent dehydration. When plate
counts were below the minimal detection level (= 10 c.f.u./ml), usually after 30 days, suspensions were considered to be non-culturable. For the infection assays, in which 40 μl suspension was used, this corresponds to 4 × 10^7 non-culturable C. jejuni/well and ≤0.4 culturable C. jejuni/well.

**INT-407 cell line, growth media and conditions**

Human embryonic intestinal cells (INT-407) obtained from the American Type Culture Collection were maintained in minimal essential medium with Earle’s salts and without glutamine (EMEM, Gibco, Life Technologies Ltd, Paisley, Scotland) supplemented with 10% heat-inactivated (30 min at 60°C) fetal bovine serum (FBS, Integro b.v., Zaandam, The Netherlands), 6 mM L-glutamine (Gibco) and 50 μg/ml gentamycin (Gibco). Cells were grown routinely in 10 ml culture medium in a 75-cm² flask (Corning Costar Europe, Badhoevedorp, The Netherlands) in a CO₂ 5% (v/v) incubator at 37°C. Confluent stock cultures were washed and released with 0.05% trypsin-EDTA and new stock cultures were seeded with 10^6 cells/ml. For the adhesion/invasion and IL-8 assays, 12-well tissue culture plates (Corning Costar Europe) were seeded with 160 000 INT-407 cells/ml per well. The plates were incubated in a CO₂ 5% (v/v) incubator at 37°C; the medium was changed three times a week. The plates were used 8 days after seeding.

**Infectivity assays**

Prior to the experiment, the medium overlaying the 8-day-old monolayers in the 12-well plates was replaced by pre-warmed EMEM, supplemented with 6 mM L-glutamine. After 1 h the cultures were inoculated with 10⁴–10⁸ culturable C. jejuni/well without or with 4 × 10⁷ non-culturable C. jejuni of the same strain per well. Bacteria were allowed to adhere to and invade INT-407 cells for 2 h in a CO₂ 5% (v/v) incubator at 37°C. After this incubation the bacteria were lysed with 1 ml 1% (v/v) Triton-X100 (Merck) in distilled water. The number of bacteria adhering to and/or invading INT-407 cells/well was determined by plating serial dilutions of the suspensions on CAB and counting the resulting colony-forming units, after 72 h incubation at 37°C under micro-aerobic conditions. Adhesion and invasion assays were performed in triplicate.

To study IL-8 secretion, 1 ml EMEM with 50 μg/ml gentamycin was added to the cells, followed by incubation for 24 h in a CO₂ 5% (v/v) incubator at 37°C. Subsequently, supernatants were collected and stored at −70°C to be analysed later. INT-407 cells without addition of bacteria were used as control. IL-8 concentrations were determined in triplicate using an IL-8 ELISA according to Garssen et al. [31].

**RESULTS**

**Adhesion and invasion**

The number of bacteria adhering to and/or invading INT-407 cells/well after infection with increasing numbers of bacteria, in a range of 10⁴–10⁸ bacteria/well, was determined for four different strains, C356, BF, 70.2, and 82/69, in the absence or presence of ±4 × 10⁷ non-culturable C. jejuni of the same strain per well (Fig.).

A similar trend was observed for all four strains: the number of bacteria adhering to and/or invading INT-407 cells/well increased as the number of C. jejuni increased until a maximum was reached, however, the curves differed per strain. The minimal needed number of culturable C. jejuni/well at which adhesion and invasion in the assay was measured, varied from ±5 × 10⁵ for C. jejuni BF to ±1 × 10⁶ for C. jejuni 70.2. The dose at which the maximal adhesion and invasion was reached, differed from ±5 × 10⁶ C. jejuni BF/well to ±1 × 10⁸ C. jejuni 70.2/well. The maximum number of bacteria adhering to and/or invading INT-407 cells, varied from ±8 × 10⁴ C. jejuni/well for strains C356, BF and 82/69 to ±2 × 10⁵ C. jejuni/well for strain 70.2.

No difference was seen between measurements in the absence or presence of non-culturable C. jejuni.

**IL-8**

The IL-8 secretion by INT-407 cells after infection with the four strains in the absence or presence of non-culturable C. jejuni of the same strain was measured (Table).

The induced IL-8 secretion varied significantly per strain, with the lowest IL-8 secretion induced by C. jejuni 70.2 and the highest IL-8 secretion...
measured after infection with C. jejuni BF. For all strains, no difference was found between measurements in the absence or presence of non-culturable C. jejuni.

**DISCUSSION**

Many conflicting articles have been written on the existence and importance of non-culturable C. jejuni.
[5–11]. To further elucidate if non-culturable C. jejuni can be infective, the effect of adding non-culturable C. jejuni on the infectivity in INT-407 cells was studied instead of measuring the effect of non-culturable C. jejuni on their own.

No differences in adhesion and invasion were found when non-culturable C. jejuni were added, which implies that non-culturable C. jejuni do not adhere or invade in vitro. However, to measure adhesion and invasion, non-culturable bacteria have to recover their culturability. If non-culturable C. jejuni can adhere or invade but cannot recover their culturability, measuring the adhesion and invasion would result in an underestimation of the infectivity, although the curves in the Figure would be affected. The curves all show an increase in the number of bacteria adhering to and/or invading INT-407 cells/well until a maximal is reached, as previously described by Biswas and colleagues [21, 32, 33].

If non-culturable C. jejuni were able to adhere or invade, but not to recover their culturability, a competition with the culturable C. jejuni would be expected. This competition between culturable and non-culturable C. jejuni would result in a decrease in the measured number of bacteria adhering to and/or invading INT-407 cells/well, which would have affected the slope of the curves in the Figure. The number of bacteria adhering to and/or invading INT-407 cells/dose and the slope of the curve were not affected, again indicating that non-culturable C. jejuni cannot adhere or invade. Furthermore, the results of the IL-8 assay which are based on measuring an immune response, also strongly support the assumption that non-culturable C. jejuni are not infective, since the addition of non-culturable C. jejuni did not affect IL-8 secretion.

Our findings strongly indicate that non-culturable C. jejuni formed at 4 °C are not infective in vitro, and conflict with the literature in which non-culturable C. jejuni formed at 4 °C were found to be infective [9–11]. Cappelier et al. [9] even reported that non-culturable C. jejuni of strains, BF and 70.2, both used in this study, were infective in two animal models. The infectivity of non-culturable C. jejuni in these former studies might be addressed by the presence of few cultivable C. jejuni. In two studies [9, 11] culturability was determined by selective enrichment. As sublethally injured C. jejuni are sensitive to selective agents, selective enrichment will negatively influence the culturability [34] and the presence of a few culturable C. jejuni might not be detected. Another explanation might be the use of in vivo instead of in vitro models, although previous studies showed a correlation between the adhesion and invasion properties and IL-8 values in vitro to the infectivity in vivo [35–37].

Our results confirm that the adhesion and invasion and the IL-8 secretion are strain dependent. The more adhesive and invasive strains, appeared to be the ones which also induced the highest levels of IL-8 in INT-407 cells, as previously shown by Hickey et al. [28, 29]. Next to strain-dependency, the level of adhesion and invasion was also found to be dose dependent as previously shown [13–15]. Our data illustrate the importance of measuring the invasion and adhesion at different doses when comparing strains. For example at a high dose ( > 8 x 10^8 C. jejuni/well) the number of bacteria adhering to and/or invading INT-407 cells was comparable for C. jejuni BF and 82/69, while at a low dose (8 x 10^9 C. jejuni/well) the number of bacteria adhering to and/or invading INT-407 cells varied by a factor of 20. C. jejuni 70.2 is in all aspects the least infective strain: the minimal dose to measure adhesion and invasion is the highest compared to the other strains, and the maximal number of bacteria adhering to and/or invading INT-407 cells is by far the lowest. The cause of the differences in infectivity between strains has not yet been elucidated.

In conclusion, our findings indicate that non-culturable C. jejuni do not adhere or invade INT-407 cells and do not induce IL-8 secretion. Therefore, assuming that the INT-407 model is comparable to the effect in vivo, the number of culturable C. jejuni in a product is a good measure for the infection risk of a product.

DECLARATION OF INTEREST

None.

REFERENCES