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

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SUMMARY

Many contradictory articles on the infectivity of non-culturable *Campylobacter jejuni* can be found. We studied the effect of non-culturable *C. jejuni* in an *in vitro* assay. To prevent the potential effect of a few culturable bacteria in the non-culturable 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-culturable *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-culturable *C. jejuni* added. These findings show that non-culturable *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-culturable *C. jejuni* can cause an infection, as in practice the exposure is often measured as the number of culturable *C. jejuni* in a product.

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In the literature many contradictory articles on the infectivity of non-culturable *C. jejuni* can be found. Some authors showed that non-culturable *C. jejuni* were not infective in chicks, mice and human volunteers [5–8] while others demonstrated that non-culturable *C. jejuni* were colonizing chicks and mice [9–11]. The inconsistency in the literature about the infectivity of non-culturable *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-culturable *C. jejuni* were formed. While some authors did not find any infectivity of non-culturable *C. jejuni* formed at 4 °C [6–8], in all studies reporting on non-culturable *C. jejuni* causing infection, the non-culturable *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 Erlenmeyer 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% O_2, 5% CO_2, 85% N_2) 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 (±10^9 *C. jejuni*/ml) were stored aerobically without shaking at 4 °C in wide-necked Erlenmeyer flasks covered with cottonwool and kitchen foil to prevent dehydration. When plate

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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 $\leqslant 0{\cdot}4$ 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-cm^2 flask (Corning Costar Europe, Badhoevedorp, The Netherlands) in a CO_2 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 10^5 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 CO_2 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 10^3–10^{10} culturable *C. jejuni*/well without or with 4×10^7 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 CO_2 5% (v/v) incubator at 37 °C. After this incubation the bacteria were removed by rinsing the monolayers three times with EMEM.

To study adhesion and invasion, the INT-407 cells 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 CO_2 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 10^3–10^{10} bacteria/well, was determined for four different strains, C356, BF, 70.2, and 82/69, in the absence or presence of $\pm 4 \times 10^7$ 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 $\pm 5 \times 10^2$ for *C. jejuni* BF to $\pm 1 \times 10^6$ for *C. jejuni* 70.2. The dose at which the maximal adhesion and invasion was reached, differed from $\pm 5 \times 10^6$ *C. jejuni* BF/well to $\pm 1 \times 10^9$ *C. jejuni* 70.2/well. The maximum number of bacteria adhering to and/or invading INT-407 cells, varied from $\pm 8 \times 10^4$ *C. jejuni*/well for strains C356, BF and 82/69 to $\pm 2 \times 10^3$ *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

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Table. *IL-8 secretion by INT-407 cells, after incubation with culturable* C. jejuni *in the absence or presence of* $\pm 4 \times 10^7$ *non-culturable* C. jejuni*/well*

Strain	Bacteria per well†	IL-8 secretion (pg/well)*	
		In the absence of non-culturable *C. jejuni*	In the presence of non-culturable *C. jejuni*
C356	$8{\cdot}2 \times 10^6$	153 ± 27	137 ± 20
BF	$7{\cdot}6 \times 10^6$	270 ± 31	284 ± 50
70.2	$8{\cdot}0 \times 10^7$	73 ± 22	79 ± 26
82/69	$9{\cdot}2 \times 10^7$	123 ± 35	106 ± 12
None		8 ± 2	—
None		—	8 ± 5‡

* All IL-8 determinations were performed in triplicate, results are means ± standard deviation.
† As undiluted overnight cultures are used, the number of bacteria added per well differs.
‡ Cocktail (1:1:1:1) of non-culturable *C. jejuni* of the four strains was used.

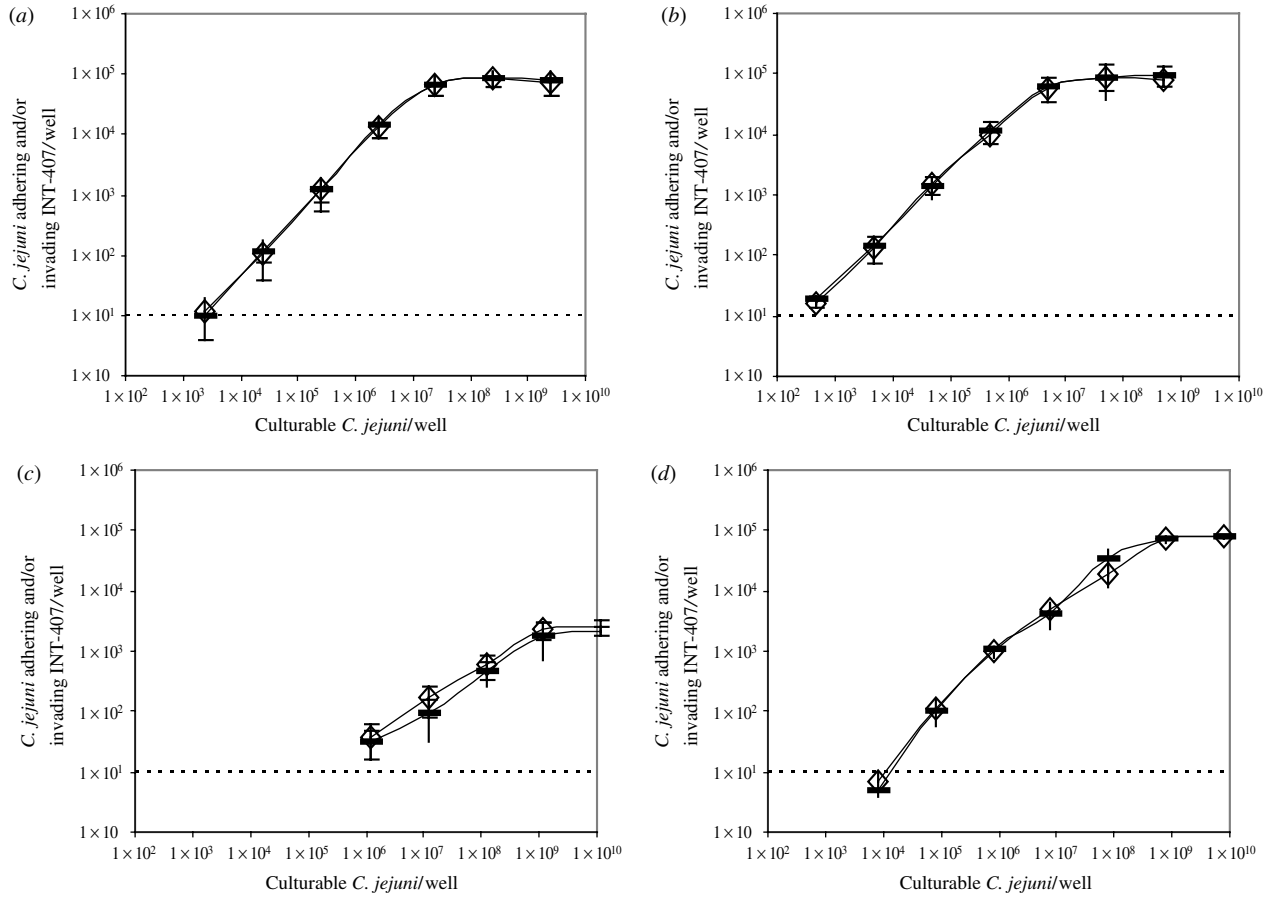

Fig. Bacteria adhering to and/or invading INT-407 cells/well after incubation with increasing numbers of culturable *C. jejuni* in the absence (–) or presence (◇) of $\pm 4 \times 10^7$ non-culturable *C. jejuni*/well. (*a*) *C. jejuni* C356, (*b*) *C. jejuni* BF, (*c*) *C. jejuni* 70.2, (*d*) *C. jejuni* 82/69. The detection limit of the assay was 10 bacteria adhering to and/or invading INT-407 cells/well.

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*

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[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 culturable *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 be not 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 ($>8\times10^8$ *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 (8×10^3 *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.

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