RESEARCH NOTE

Detection of *Campylobacter jejuni* Invasion of HEp-2 Cells by Acridine Orange-Crystal Violet Staining

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Since the identification of *Campylobacter jejuni* as an enteropathogenic agent, it has been shown that the clinical picture of *Campylobacter* enteritis suggests the existence of an invasive mechanism in this bacteria (R Walker et al. 1986 *Microbiol Rev 50*: 81-94). The ability of *C. jejuni* to invade epithelial cells was confirmed *in vivo* using chicken (L Field et al. 1986 *Infect Immun 54*: 118-125), mice (M Youssef et al. 1987 *Infect Immun 55*: 1019-1021), hamsters (C Humphrey et al. 1985 *J Infect Dis 152*: 485-493), and monkeys (R Russel et al. 1989 *Infect Immun 57*: 1438-1444) as biological models. Their *in vitro* invasion potential has been demonstrated in mammalian cell lines using electron microscopy (M de Melo et al. 1989 *Infect Immun 57*: 2214-2222), indirect immunofluorescence techniques (M Konkel & L Joens 1989 *Infect Immun 57*: 2984-2990), radiolabelled bacteria (G Lindblom et al. 1990 *APMIS 98*: 179-184) and May Grünwald Giemsa staining (H Fernández & L Trabulsi 1995 *Biol Res 28*: 205-210). Most of the latter techniques are cumbersome or difficult to be carried out or even difficult to read and interpret. Recently, the use of an acridine orange staining technique combined with crystal violet counterstaining (AO-CV stain) was proposed to investigate the presence of several intracellular enteropathogens other than *Campylobacter* in HeLa cells (M Milliotis 1991 *J Clin Microbiol 29*: 830-831).

In the present study we used this technique to demonstrate invasiveness of *C. jejuni*, by employing transmission electron microscopy (TEM) as standard control.

Twenty *C. jejuni* strains were tested for *in vitro* invasiveness. The source of the strains is shown in Table. *In vitro* invasiveness tests were carried out as previously described (Fernández & Trabulsi *loc. cit*.) but using AO-CV stain (Milliotis *loc. cit*.). In brief, HEp-2 cells were cultured overnight on coverslips (20 x 8 mm), in Leighton tubes containing minimum essential medium with 10% fetal calf serum (MEM-10%), at 37ºC under 5% CO_2 atmosphere. After washing three times with PBS, the medium was replaced with 1 ml *C. jejuni* suspension ($6x10^8$ colony forming units) in MEM with 2% fetal calf serum. Cells were incubated for 3 hr at 37ºC and 5% CO_2 (infection period), washed 10 times in PBS and reincubated for 4 hr with 1 ml MEM-10% (multiplication period). Then, coverslips were washed three times with PBS and, without fixing, stained with 0.01% acridine orange in Gey's solution for 45 sec, rinsed with Hanks balanced salt solution and counterstained with 0.05% crystal violet in 0.15 N NaCl for 45 sec, mounted on slides and sealed with colorless nail polish and examined under epi-fluorescence microscopy at 400X magnification for screening and 1000X magnification for quantitative evaluation. The invasion rate (no. of invaded cells/total cells examined x 100) was determined by counting at least 200 cells. The average number (± SD) of invading bacteria was estimated in a minimum of 35 invaded HEp-2 cells. All tests were carried out twice in duplicate.

Five invasive and two non-invasive strains were randomly selected for TEM as control tests. Invasion tests were done as described above but using cell monolayers in 35 mm tissue culture dishes. After the multiplication period, the cell monolayers were washed three times with PBS and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 hr, gently scraped and washed with the same buffer under low speed centrifugation. Cell pellets were placed in agar and small blocks were cut and prepared for TEM as described previously for *Escherichia coli* by U Fagundes-Neto et al. (1995 *Acta Paediatrica 84*: 453-455).

Results are reported in Table. Sixteen of the 20

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TABLE

Invasion of HEp-2 cells by *Campylobacter jejuni* studied with an acridine orange-crystal violet staining (AO-CV staining) and transmission electronic microscopy (TEM)

		AO-CV staining		
Strain	Origin	Invasion rate	Nº bacteria/cell	TEM invasion
1	Human[a]	16.0	4.9 ± 2.1	ND
2	Human[a]	21.0	12.5 ± 5.5	+
3	Human[a]	23.5	9.0 ± 4.8	+
4	Human[a]	18.0	10.3 ± 3.2	+
5	Human[a]	21.0	12.5 ± 5.6	+
6	Human[a]	23.5	15.4 ± 9.2	ND
7	Human[a]	16.0	7.3 ± 3.9	ND
8	Human[a]	NI		NI
9	Human[a]	NI		ND
10	Pork	8.5	5.1 ± 1.9	ND
11	Pork	24.0	10.1 ± 6.4	ND
12	Pork	18.5	14.2 ± 6.5	ND
13	Pork	NI		ND
14	Sparrow	8.0	6.3 ± 3.1	ND
15	Sparrow	8.5	6.7 ± 6.0	ND
16	Sparrow	NI		NI
17	Duck	42.0	38.2 ± 19.2	+
18	Duck	14.5	10.3 ± 7.7	ND
19	Chicken liver	15.5	8.8 ± 7.1	ND
20	Chicken liver	19.5	9.2 ± 7.8	ND

a: isolated from human cases of diarrhea; NI: non invaders; ND: not done

C. jejuni strains studied were able to invade HEp-2 cells. In each case, using the AO-CV stain, it was possible to observe intra-cytoplasmatic curved bright green-fluorescing bacteria into the HEp-2 cells. The invasion rate ranged from 8% to 42% whereas the number of bacteria per invaded HEp-2 cell varied from 4.9 ± 2.1 to 38.2 ± 19.2. Fig. 1a shows typical forms of *Campylobacter* inside the cytoplasm of a Hep-2 cell.

Different techniques have been used to study *C. jejuni* invasiveness and since the obtained results are not uniform, they do not allow effective or valid comparisons. In our study, invasion by *C. jejuni* was assessed by a double-stain technique (AO-CV stain) that allows the optical observation (fluorescence microscopy) of internalized bacteria exclusively. This is an advantage of this method since it is difficult to diferentiate extra and intracellular bacteria by employing other methods. The rate of invasion and the number of bacteria per invaded cell observed with this technique are in agreement with a previous report in which the same infection and multiplication periods were used to investigate invasion by May Grünwald-Giemsa staining (Fernández & Trabulsi *loc. cit.*). G Bukholm and G Kapperud (1987 *Infect Immun 55*: 2816-2821), using a combination of Nomarski differential interference contrast microscopy and UV incident-light microscopy to discriminate between extra and intracellular bacteria, showed that *Campylobacter* strains, having *Salmonella typhimurium* as a coinfectant, could invade cell cultures similarly to that observed with the AO-CV stain technique employed in this study.

The ability to invade, demonstrated by five strains observed with acridine orange-cristal violet staining method, was confirmed by TEM. In all TEM control tests carried out with the invasive strains, but not with the non-invasive ones, it was possible to observe intracellular curved bacteria that seemed to be enclosed into a vacuole, as shown in the Fig. 1b. Similar observations were previously reported by de Mello et al. (*loc. cit.).*

These results suggest that the acridine orange-crystal violet staining is a suitable technique to study *in vitro* the ability of *C. jejuni* to invade cell cultures, similarly to other bacteria as it was previously demonstrated for *Arcobacter cryaerophylus* by H Fernández et al. (1995 *Mem Inst Oswaldo Cruz 90*: 633-634).

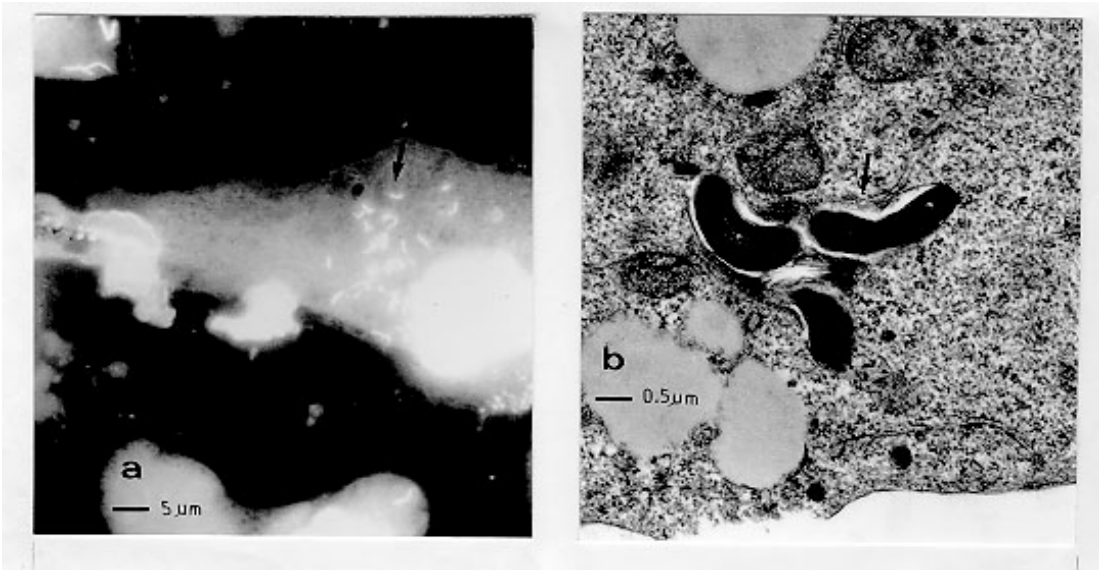

Fig. 1: a - Fluorescent internalized campylobacters (arrow) into HEp-2 cells observed with acridine orange-crystal violet staining. Bar = 5 µm. b - Internalized campylobacters (arrow) within cytoplasmic vacuoles observed by transmission electron microscopy. Bar = 0.5 µm.