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Brief Communication

In vitro Invasion of *Aeromonas* spp. to HEp-2 Tissue Culture Cells

Epithelial cell invasion of certain pathogenic bacteria is an important step in the process of pathogenesis (*Formal et al.* 1983). The critical event in the onset of overt infection of *Shigella, E. coli, Yersinia* and *Salmonella* is their capacity to invade and penetrate into the host cells (*Formal et al.* 1983, *Une* 1977, *Kihlström* 1977). This invasion is a predominant virulence property of these pathogens (*Formal et al.* 1983). In the case of *Aeromonas* spp. there are few reports on their invasion of epithelial cells. *Lawson et al.* (1985) and *Watson et al.* (1985) have reported on the invasive property of *Aeromonas* spp. isolated from humans.

Bacterial invasiveness can be demonstrated either by testing in the guinea pig eye (Serény test: *Serény* 1955) or in tissue culture cells such as HeLa or HEp-2 cells (*Kihlström* 1977, *Lawson et al.* 1985).

This study was aimed at investigating the invasive potential of animal isolates of *A. hydrophila* and *A. sobria* using the HEp-2 tissue culture cells. Invasion of *Aeromonas* was detected by light and electron microscopy (EM) techniques.

Strains of *Aeromonas* spp. were isolated from fish and hare (Table 1) and were stored at –70°C in Trypticase Soya Broth (TSB, Difco) containing 15 % glycerol. Strains were cultured on blood agar (5 % horse blood) and incubated at 20°C for 24 h. The bacteria were later harvested and inoculated into 100 ml Trypticase Soya Broth (TSB) and incubated at 20°C on a shaker (60 rpm) for 20 h. The cultures were centrifuged (16,000 xg) at 4°C for 30 min. The cell pellets were resuspended in sterile phosphate buffered saline (PBS, 10 mmol/l sodium phosphate, pH 7.4 containing 0.14 mmol/l NaCl) and different dilutions of bacterial suspensions were used for invasion studies.

HEp-2 (human epidermal carcinoma, Flow Laboratory, Irvine, Scotland) cells were maintained in basal medium eagle (BME; Flow) containing 10 % foetal calf serum, 50 units penicillin-G and 50 µg of streptomycin/ml. Cells were incubated at 37°C in an atmosphere containing 5 % CO_2. For experimental purposes the cells were seeded on glass cover slips placed in sterile petri dishes containing growth culture medium and allowed to grow into monolayers.

Two hours before infection the HEp-2 monolayers cultured on glass cover slips were washed twice with BME media without antibiotics to get rid of the antibiotics in the cell culture medium. The HEp-2 cell monolayers in BME media (without antibiotics) were infected with the respective bacterial cultures diluted in 0.145 M NaCl to a final concentration of 10^6–10^7 bacteria/ml. After an infection period of 2 h at 20°C the cover slips were washed (5 times or more) with PBS to minimize the presence of extracellular bacteria. The cover slips were then placed in petri dishes with fresh BME media supplemented with 50 µg/ml gentamycin (Shering Co., New Jersey, USA) to protect the cells from reinfection by extracellular live bacteria and to eliminate the cell-surface attached bacteria. After 5 h of incubation at 20°C the cover slips were washed as described above, fixed by 100 % methanol

and stained with crystal violet for 1 min. The cover slips were then examined by light microscope.

To detect the intracellular bacteria by scanning EM the HEp-2 cell monolayers, grown on cover slips and infected with bacteria for a total period of 7 h at 20°C were fixed *in situ* with 1.5 % glutaraldehyde in PBS. The specimens were rinsed with PBS, postfixed in 1 % osmium tetraoxide (OsO_4) in distilled water and rinsed in the same buffer. They were then dehydrated in graded series of acetone concentrations and dried in critical point drying apparatus (Balzer 010). The cover slips were mounted on stubs, coated with carbon and gold, and examined by scanning electron microscope (JOEL-ISM820) for the presence of intracellular bacteria.

To detect intracellular bacteria by transmission EM the HEp-2 cell monolayer on cover slips (infected with bacteria as above) were detached by 0.25 % Trypsin (3 min at 20°C). Detached cells were fixed in OsO_4 as described above. The cells were then dehydrated in graded series of ethanol concentrations and transferred to Beem-capsules and embedded in Epon. The sections were cut on LKB ultramicrotome and examined for the presence of intracellular bacteria in Philips 420 transmission electron microscope.

The light and electron microscopic examinations showed that some of the investigated *A. hydrophila* and *A. sobria* strains isolated from fish were invasive in HEp-2 cell monolayers. Two of the 3 *A. hydrophila* strains investigated, had the ability to penetrate and invade the HEp-2 cell monolayer (Table 1). Interestingly, 1 of these invasive strains (10–74) was isolated from a hare. In addition, 2 strains of another motile *Aeromonas* namely *A. sobria* (isolated from fish) were also included in these experiments and 1 of these strains had the capacity to invade the HEp-2 cells (Table 1). When the infected HEp-2 cells were observed under light microscope (Fig. 1) the number of intracellular bacteria ranged approximately between 6–15 organisms/cell. Furthermore, the electron microscope observations showed that bacteria were entrapped by the HEp-2 cell micro-

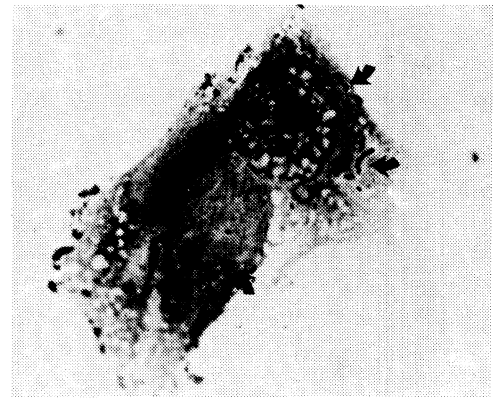

Figure 1. Invasion of HEp-2 cells by *A. hydrophila* strain 10–74 observed under light microscope. Arrows indicate the invasive organisms enclosed in the cell vesicles (magnification 1000 ×).

Table 1. Invasion of HEp-2 cells by *Aeromonas* spp.

Strain	Isolated from	Invasion	No. of intracellular bacteria/cell
A. hydrophila 70-4	fish	–	0
A. hydrophila 1865	"	+	5–>12
A. sobria 34-74	"	+	5–> 12
A. sobria 29-74	"	–	0
A. hydrophila 10-74	hare	+	6–>15

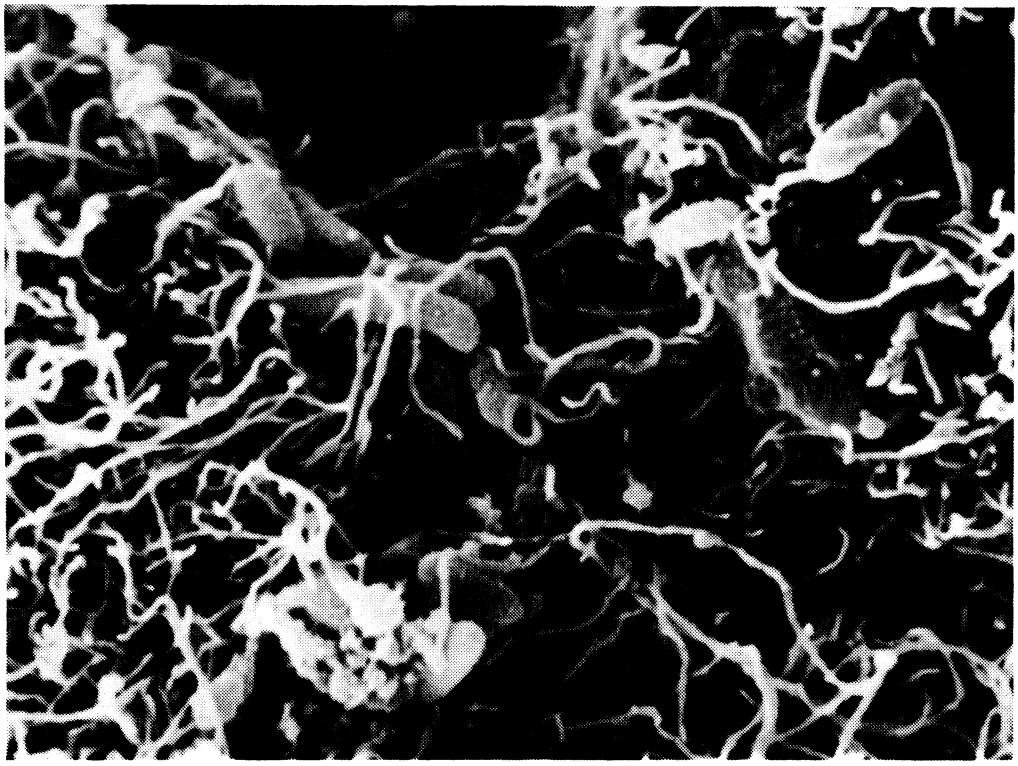

Figure 2. Electron microscograph showing cells of *A. hydrophila* strain 1865 engulfed in the HEp-2 cell microvilli (magnification 43,000 ×).

villi (Fig. 2) and penetrated the HEp-2 cells (Fig. 3).

The role of *Aeromonas* spp. as human pathogen has increasingly become evident during the last two decades. Human gastroenteritis caused by *A. hydrophila* has been recognized on a world-wide basis and the increasing frequency of its isolation has also been reported (*Janda et al.* 1988). Moreover, *Aeromonas* spp. were early recognized as pathogens for cold-blooded animals (*Sanarelli* 1891) and have also been shown to be of etiological importance in cultured and wild marine fish disease (*Austin & Austin* 1987). It is well known that *Aeromonas* may produce a wide range of extracellular products such as cytotoxin, enterotoxin, protease, elastase which have been implicated as potential virulence factors for humans and/or fish (*Ljungh & Wadström* 1986, *Austin & Austin* 1987). These factors could be involved in the enhancement of invasion of these pathogens. The invasion of *Aeromonas* spp. into the host may be an additional virulence factor in the mechanism of pathogenicity of these organisms, in humans as well as in fish.

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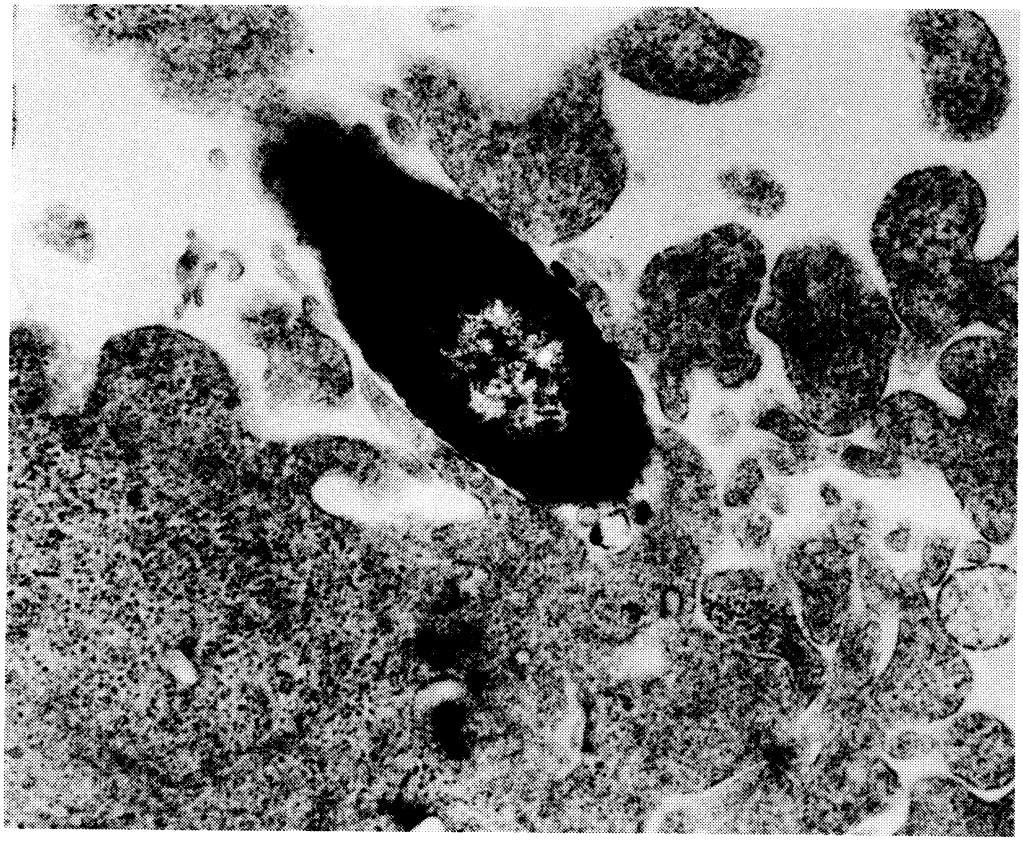

Figure 3. Transmission electron microscograph showing the penetration of *A. hydrophila* strain 1865 in HEp-2 cells. Note the destruction of the epithelial cell (magnification 55,000 ×).

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Karel Krovacek, Ahmed Faris and
Ingmar Månsson
Section of Bacteriology and Epizootology,
Department of Veterinary Microbiology,
Faculty of Veterinary Medicine,
Swedish University of Agricultural Sciences,
Uppsala, Sweden.

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Reprints may be requested from: Karel Krovacek, Section of Bacteriology & Epizootology, Biomedicum, Box 583, Uppsala, S-75 123, Sweden.