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Short Communication

Interaction of *Yersinia enterocolitica* Biotype 1A Strains of Diverse Origin with Cultured Cells In Vitro

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SUMMARY: *Yersinia enterocolitica* biotype 1A isolates are increasingly being associated with diarrhea. However, the mechanism of their pathogenicity is not well understood. In the present study interaction of *Y. enterocolitica* isolates with CHO cells, HEp-2 cells, and J774 mouse macrophages was studied. *Y. enterocolitica* biotype 1A strains of clinical origin invaded CHO and HEp-2 cells to a significantly higher degree than non-clinical isolates. However, among non-clinical isolates, *Y. enterocolitica* strains of swine origin showed significantly more invasion in CHO and HEp-2 cells than water isolates. *Y. enterocolitica* isolates from clinical samples exhibited a greater level of survival in macrophages than isolates from non-clinical sources. It may be construed that *Y. enterocolitica* biotype 1A isolates of clinical and swine origin have higher virulence potential than those from other sources.

Yersinia enterocolitica, an important food- and water-borne enteric pathogen, is associated with various clinical manifestations ranging from self-limited gastroenteritis to more invasive syndromes such as terminal ileitis and mesenteric lymphadenitis (1). *Y. enterocolitica* is extremely heterogeneous biochemically and pathogenically, and is represented by six biotypes: 1A, 1B, 2, 3, 4, and 5. Strains belonging to biotypes 1B and 2-5 invade tissue-cultured cells in large numbers, resist phagocytosis and induce apoptosis in these cells (1,2). These invasive strains are also known to cause disease of varying severity in human beings and animals. Biotype 1A strains, however, have been reported to lack these markers of the pathogenicity of *Y. enterocolitica* (2,3). Consequently, in the past these strains were regarded as avirulent. Nevertheless, biotype 1A strains have been implicated in nosocomial and milk-borne outbreaks in several parts of the world (4-6). Also, a significant proportion of the *Y. enterocolitica* isolated from patients with diarrhea belong to biotype 1A and the gastrointestinal illness associated with them was reported to be indistinguishable from that caused by the primary pathogenic biotypes (7-9).

It has been hypothesized that some subsets of biotype 1A strains of *Y. enterocolitica* may have the ability to cause disease. A recent study showed that biotype 1A strains of clinical origin invaded tissue-cultured epithelial cells and resisted killing by macrophages to a significantly greater extent than the non-clinical isolates (10,11). However, interaction of *Y. enterocolitica* biotype 1A strains isolated from swine – the major source of *Y. enterocolitica* – and other sources with tissue-cultured cells is not well understood. In a study conducted in Delhi, India, we isolated *Y. enterocolitica* from diverse sources, viz., stools of diarrheic patients, swine throats, groundwater, river water, and wastewater (12). All isolates belonged to biotype 1A. The aim of the present study was to investigate the interaction of these isolates with cultured cells in vitro.

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Thirty-six strains of *Y. enterocolitica* biotype 1A, which comprised 18 strains isolated from diarrheic stools of pediatric patients, 11 from swine throat and 7 from various water sources (river, groundwater, and wastewater) were used in this study. Details of these strains have been reported earlier (12). The clinical isolates were of serotypes O:6,30 and O:6,30-6,31, while the aquatic isolates belonged to serotype O:10-34 (12). Further analysis of these strains using specific probes revealed that the majority harbored gene B (*yst B*) for *Yersinia* stable toxin (13). Two virulence plasmid (pYV)-bearing strains of *Y. enterocolitica* IP 26332 (biotype 2, serotype O:3) and IP 26249 (biotype 2, serotype O:5,27) obtained from *Yersinia* National Reference Laboratory and WHO Collaborating Center, Pasteur Institute, Paris were also used. In addition, *Escherichia coli* HB101 was used as a negative control. Strains of *Y. enterocolitica* and *E. coli* were grown routinely in BHI broth (Hi Media, Mumbai, India) at 28°C and 37°C, respectively.

HEp-2 cells were grown in minimal Eagle's medium (MEM) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine and 20 mM HEPES (Hi Media). CHO cells were cultured in MEM containing 10% FBS, glutamine and HEPES as described above. Similarly, J774 mouse macrophages were grown in RPMI-1640 medium supplemented with FBS, glutamine and HEPES as mentioned earlier. All cells were grown at 37°C in a humidified atmosphere under 5% CO_2. Twenty hours prior to the infection, approximately 4×10^5 cells were seeded per well of 6-well tissue culture plates (Greiner, Wemmel, Belgium).

Bacteria were grown in BHI broth at 28°C for 12 h and added to the cell monolayer at a multiplicity of infection (MOI) of 50:1. To determine the invasiveness of bacteria, at 3 h of infection, HEp-2 and CHO cells were washed thrice with PBS and incubated further for 90 min in MEM containing 100 μg/ml of gentamicin. Thereafter the cells were washed twice with PBS to remove gentamicin. The number of the intracellular bacteria was determined by lysing the cells in PBS containing 0.1% digitonin and plating on trypticase soy agar (TSA). To determine the extent to which bacteria replicated in HEp-2 and CHO cells, in a parallel series of wells, the medium containing 100 μg/ml of gentamicin was

replaced with medium containing 10 μg/ml of gentamicin. After 18 h cells were lysed with digitonin and the number of bacteria was determined on TSA.

To measure bacterial survival in macrophages at 1 h post-infection, the cells were washed twice with PBS, and fresh RPMI-1640 medium containing 100 μg/ml of gentamicin was added for 30 min to kill the extracellular bacteria. To determine the number of bacteria phagocytosed, cells were lysed and bacteria enumerated on TSA. To determine the bacterial survival in macrophages, in a parallel series of wells, the RPMI-1640 medium containing 100 μg/ml of gentamicin was replaced with medium containing 20 μg/ml of gentamicin and incubated for 24 h. Thereafter, the cells were lysed and the intracellular bacteria enumerated.

To determine the cell viability, HEp-2, CHO, and the macrophage cell lines were infected with bacteria as in the assays for bacterial replication or survival. The viability of tissue-cultured cells was determined by their ability to exclude trypan blue. The cells were stained with 0.1% of trypan blue for 5 min and observed for dye exclusion with an inverted microscope.

The results were analyzed by Student's *t* test. Values of $P < 0.05$ were considered to indicate statistical significance.

The results of the invasion assay are presented in Table 1. Approximately 0.015 to 1.2% of the total bacteria of clinical isolates (strains isolated from pediatric patients) of *Y. enterocolitica* biotype 1A invaded CHO cells. In contrast, swine and water isolates recovered from CHO cells had a rate of invasion of 0.009 to 0.23 and 0.002 to 0.012%, respectively. The virulence plasmid-bearing strains of *Y. enterocolitica* IP 26332 and IP 26249 exhibited levels of invasion of 3.2 and 2.7%, respectively.

All 36 *Y. enterocolitica* biotype 1A isolates showed lower levels of invasion into HEp-2 cells than into CHO cells (Table 1). By comparison, in both HEp-2 and CHO cells, clinical isolates of *Y. enterocolitica* showed a significantly ($P < 0.05$) higher level of invasion than swine or water isolates. Finally, *Y. enterocolitica* strains of swine origin showed a significantly ($P < 0.05$) greater level of invasion than water isolates.

All *Y. enterocolitica* biotype 1A isolates showed replication inside CHO and HEp-2 cells, as evidenced by the 1.7- to 5.1-fold increase in the number of bacteria recovered after 18 h of culturing compared to the number detected in the invasion assay (Table 1). However, the increase in the numbers of strains of *Y. enterocolitica* isolated from various sources was not statistically significant ($P > 0.05$).

Among clinical isolates of *Y. enterocolitica*, 19.5 to 82.4% of bacteria phagocytosed by J774 macrophages survived after 24 h (Fig. 1). For the swine and water isolates, the survival rate varied from 18.7 to 39.4 and 14.9 to 18.8%, respectively, indicating that the survival of clinical isolates was significantly ($P < 0.05$) greater than that of the swine and water isolates. Only 9.1% of *E. coli* HB101 phagocytosed by macrophages were recovered after 24 h. Survival of the virulence plasmid-bearing strains in macrophages could not be determined because of their cytotoxicity to cultured cells.

The integrity of the cultured cells was examined by their ability to exclude trypan blue. More than 99% of the macrophages, HEp-2 and CHO cells excluded trypan blue when incubated with *Y. enterocolitica* biotype 1A isolates. By contrast, incubation of cells with virulence plasmid-bearing *Y. enterocolitica* strains IP 26332 and IP 26249 resulted in 70 to 75% cell viability. For *E. coli* HB101 the viability of the cells was always more than 98%.

Table 1. Invasion and replication of *Y. enterocolitica* isolates in CHO and HEp-2 cells

Isolate	Percent* invasion		Replication* (times increase)	
	CHO cells	HEp-2 cells	CHO cells	HEp-2 cells
C 20	0.091	0.026	2.4	1.9
C 93	0.466	0.018	2.8	3.6
C 770	0.387	0.025	2.1	2.5
C 927	0.281	0.042	3.2	3.4
C 112	0.230	0.011	2.6	3.1
C 945	0.572	0.029	2.1	2.5
C 51	0.280	0.013	3.2	2.1
C 801	0.299	0.010	2.3	3.9
C 1021	1.100	0.053	2.8	3.1
C 94	0.403	0.031	3.0	2.8
C 114	1.200	0.050	3.9	3.3
C 792	0.130	0.009	3.8	4.5
C 871	0.096	0.022	2.2	3.2
C 963	0.180	0.008	4.6	3.2
C 782	0.020	0.001	2.2	2.5
C 842	0.015	0.0009	2.6	2.2
C 845	0.028	0.0008	2.8	2.9
C 791	0.026	0.0011	3.4	3.5
P 249	0.090	0.013	4.2	4.8
P 307	0.230	0.020	3.1	2.9
P 334	0.021	0.007	2.1	5.0
P 290	0.090	0.004	2.1	4.8
P 395	0.092	0.005	3.6	4.6
P 456	0.108	0.011	2.6	2.3
P 204	0.012	0.0009	3.2	2.1
P 215	0.015	0.0009	3.4	2.7
P 466	0.009	0.0006	5.1	4.1
P 400	0.020	0.0011	2.1	2.3
P 437	0.017	0.0009	2.9	2.6
G 230	0.009	0.0006	4.3	2.6
W 17	0.008	0.0	2.0	0
G 116	0.008	0.0	1.8	0
G 196	0.002	0.0	1.7	0
W 123	0.012	0.0008	1.9	2.2
W 194	0.009	0.0006	2.3	1.8
R 31	0.007	0.0	2.7	0

*: Values are mean of at least three experiments.
C, clinical; P, swine; G, groundwater; W, waste water; R, river water isolates.

Y. enterocolitica biotype 1A strains are increasingly being associated with diarrhea (5,8,9). However, the mechanism of their pathogenicity is not well understood. Invasion into cells, and intracellular survival are important determinants of the virulence of primary pathogenic biotypes of *Y. enterocolitica* (1). Recently, Grant et al. (10,11) reported that *Y. enterocolitica* biotype 1A strains of clinical origin invaded CHO and HEp-2 cells to a greater extent than non-clinical isolates. In the present study also, it was observed that biotype 1A isolates from diarrheic patients invaded cultured cells to a higher degree than non-clinical isolates. An important finding of the present study, however, was that among the non-clinical isolates, strains of swine origin showed a significantly higher level of invasion than isolates from water. Moreover, a number of the isolates of swine origin showed levels of invasion into CHO and HEp-2 cells in vitro that were similar to those of clinical isolates. These observations indicated that *Y. enterocolitica* biotype 1A isolates of swine origin had greater pathogenic potential than those of water. This finding was further supported by the observation that strains from swine

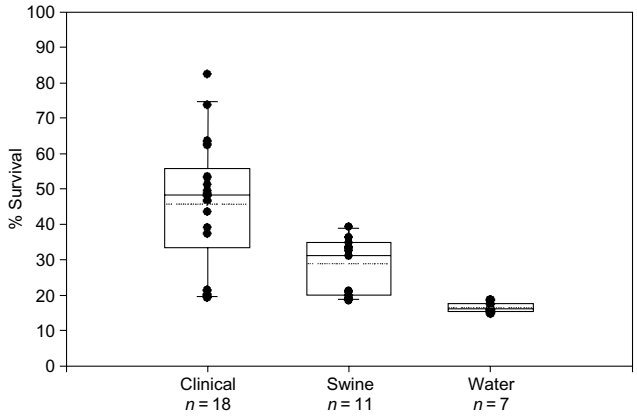

Fig. 1. Survival of *Y. enterocolitica* isolated from various sources inside J774 murine macrophages. The limits of the boxed area are the 25th and 75th percentiles. Within the box, the solid line marks the median and the dotted line represents the mean. The small bars represent the 10th and 90th percentiles. Each point represents the value for an individual strain.

showed relatively greater survival in J774 macrophages than isolates from water. *Y. enterocolitica* biotype 1A strains are known to be poorly equipped to survive in their hosts due to their susceptibility to killing by complement and polymorphonuclear leukocytes (11,14). Thus, the ability of *Y. enterocolitica* biotype 1A to sequester themselves within host cells may be an important marker of their pathogenicity. The ability of bacteria to survive within macrophages may aid in their persistence in the host.

Swine are generally regarded as the major reservoir of pathogenic *Y. enterocolitica*, since the serotypes most commonly involved in human infections are carried in the oral cavity of healthy pigs (15). The pathogenic potential of *Y. enterocolitica* biotype 1A isolated from swine further stresses the importance of this animal as a source of infection. The finding that the isolates of clinical and swine origin showed a higher level of invasion and better survival in cultured cells than those isolated from water may have been due to potentiation of these isolates by colonization of human and swine hosts. The mechanisms underlying such potentiation may be a key to understanding the pathogenicity of biotype 1A isolates of *Y. enterocolitica*.

In conclusion, the present study suggests the existence of heterogeneity in the pathogenicity of biotype 1A strains of both clinical and swine origin, i.e., some clinical isolates may be non-pathogenic, and some isolates from swine may have the potential for virulence. Thus, the potential for pathogenicity of *Y. enterocolitica* biotype 1A isolates from non-clinical sources, particularly swine, should not be completely disregarded.

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