Experimental Kinetics of Infection Induced by *Yersinia pseudotuberculosis* Isolated from Stock Animals

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The course of in vivo infection of five isolates of Yersinia pseudotuberculosis *was followed for three weeks in Swiss mice. The strains were isolated from diarrheic and normal feces and mesenteric lymph nodes of healthy and sick stock animals. Four strains of serogroup O:3 and one of serogroup O:1a, with and without the virulence plasmid, were inoculated intragastrically and intravenously in the mice. Groups of five animals were sacrificed at 6 h and 3, 6, 10, 15, and 21 days after inoculation, and organs and tissues were checked for possible macroscopic alterations. Development of infection was monitored at these times by performing viable bacterial counts in homogenates of selected tissues. The animals were cheked daily for clinical alterations. The results of the study showed that strains with the virulence plasmid infected organs and tissues at various times and at varying intensity by both routes of infection, the strain of type O:1a being the most invasive. Moreover, clinical and pathological alterations occurred only in animals inoculated with bacteria carrying the virulence plasmid, regardless of the route of infection.*

Key words: *Yersinia pseudotuberculosis* - kinetics of infection - virulence

Yersinia pseudotuberculosis causes various clinical syndromes in men and animals. To induce disease, a set of virulence factors related to plasmid and chromosomal genes must be present, which contribute to host colonization and prevent the action of specific and nonspecific host defense mechanisms.

Pathogenic *Y. pseudotuberculosis*, like other pathogenic *Yersinia* species, harbors a plasmid of 70-75 kb, known as the *Yersinia* virulence plasmid pYV (Brubaker 1991). The products of the genes on this plasmid are grouped into four main categories: adhesion/invasion protein (YadA), antiphagocytic secreted proteins (Yops), proteins involved in Yop processing and secretion (Ysc), and regulatory proteins (Lcr) (Salyers & Whitt 2002). Among the latter is the LcrF protein. Invasion protein (Inv) coded by chromosomal gene is also one of the virulence mechanisms of pathogenic *Yersinia*. Invasiveness is mediated by gene *inv* on the chromosome and gene *yadA* on the plasmid (Revell & Miller 2001). Another very important characteristic of *Yersinia* virulence mediated by chromosomal genes is the ability to capture iron, which is regulated by a set of genes forming the high-pathogenicity island, HPI (Carniel 1999).

Interaction between bacteria and host cells is an important step in the establishment of infection. After intestinal colonization by pathogenic *Yersinia*, the bacteria penetrate the intestinal mucosa through the M cells. After penetration, *Yersinia* colonize Peyer's patches, possibly disseminating through the lymphatic pathway and eventually reaching the mesenteric lymph nodes, the liver, and the spleen (Grutzkau et al. 1990, Falkow et al. 1992, Clark et al. 1998).

The pathogenicity of *Yersinia* can be studied in vitro and in vivo; in vitro by searching for the different genes involved or their products and in vivo by experimental infection and monitoring its kinetics in animals such as mice, rats, rabbits or guinea pigs. The latter has been well documented in *Y. enterocolitica* (Quan et al. 1974, Carter & Collins 1974, Alonso et al. 1975, 1978, Une 1977, Falcão et al. 1984, Apfel & Noleto 1991, Bauab & Falcão 1991, Heesemann et al. 1993). However in *Y. pseudotuberculosis,* only a few studies have shown in vivo infection, and the kinetics has not been studied.

Working with *Y. pseudotuberculosis* O:3 strains harboring the virulence plasmid and inoculated by the oral (5×10^9 bacteria/ml) and intraperitonial (from 10^4 to 10^7 bacteria/ml) routes in BALB/cJ mice, Holmström et al. (1995) observed systemic infection in all animals.

After inoculating guinea pigs by the respiratory route (in an aerosol chamber of the flow-dynamic type) with *Y. pseudotuberculosis* (nr 5763, Czechoslovac Collection), Veljanov et al. (1993) described the involvement of the lungs and tracheobronchial organs of animals in early infection, which later became generalized. The infectious process was monitored on the basis of the increased numbers of polymorphonuclear leukocytes and phagocytic activity.

After inoculating mice intraperitoneally (10^7 organisms) with melibiose-fermenting and non-fermenting *Y. pseudotuberculosis* O:3 strains isolated from healthy swine, Tsubokura et al. (1984) correlated the death of mice with the ability of the bacteria to ferment the carbohydrate.

This study investigates the kinetics of infection induced in mice by *Y. pseudotuberculosis* strains with or without some plasmidial and chromosomal virulence markers.

MATERIALS AND METHODS

Bacterial strains - A total of five *Y. pseudotuberculosis* strains isolated from stock animals in Brazil were studied. Their previously determined characteristics (Martins et al. 1998), are shown in the Table.

Experimental infection - Performed according to Falcão et al. (1984).

For each experiment, 35 female Swiss mice aged five to six weeks were used. Bacteriologically-controlled animals were obtained from the Central Animal House of São Paulo State University (Unesp) at Botucatu. Groups of five animals were kept in disinfected cages with free access to previously sterilized food and water. To activate the virulence of the bacteria, 0.5 ml of a 10^8 cells/ml saline suspension of the strain, grown in blood agar base for 24 h at 25°C, was inoculated intravenously into two mice. After 24 h, the animals were killed and their spleens removed. A saline homogenate of each spleen was poured on to blood agar base plates and incubated at 25°C for 48 h. The growth on these plates was used to prepare the inoculum. A selected smooth colony was streaked on the surface of a blood agar base plate and incubated at 25°C for 24 h. The resulting growth was harvested in 10 ml of sterilized saline. Two suspensions were prepared from this material to obtain inocula with 10^8 cells/ml for intragastric inoculation (IG) and 10^6 cells/ml for intravenous (IV) inoculation (Falcão et al. 1984, Bauab & Falcão 1991). Infection was induced either with a 0.5 ml suspension (10^6 cells/ml) injected into the tail vein on with a 0.25 ml suspension (10^8 cells/ml) administered intragastrically.

Groups of five mice were sacrificed 6 h and 3, 6, 10, 15, and 21 days after inoculation. The remaining five mice were kept to complete the groups, in the case of death of animals before the end of the experiments on the 21st day. One-third of the cecum, the liver, spleen, and mesenteric lymph node chain were removed from each mouse, four Peyer's patches from the small intestine were excised and 0.5 ml of blood was obtained by cardiac puncture. 0.1 ml of the blood was immediately surface-plated on blood agar base.

The cecal content was homogenized in nutrient broth and all the others in saline. All homogenates were diluted in saline (10^{-1} - 10^{-3}). Peyer's patches were washed three times in cold saline prior to homogenization and dilution. A 0.1 ml aliquot of each tenfold dilution in saline was surface-plated in duplicate on blood agar base. The colony-forming units were counted up to 48 h of incubation at 25°C. *Y. pseudotuberculosis* colonies were confirmed by slide agglutination using specific antiserum.

Clinical and pathological alterations - During the entire experiment, live animals were observed daily for clinical alterations such as diarrhea, prostration, emaciation, ruffled hair, etc. After being killed, they were examined for lesions and other visible pathological manifestations.

RESULTS

The course of in vivo infection of each of the five strains of *Y. pseudotuberculosis* is represented graphically in Figs 1-5.

The Yp36 strain, of serogroup O:3 but without plasmid pYV, when inoculated by the IG route (Fig. 1), was found 6 h later in the cecal content and Peyer's patches, and 3 days later in the mesenteric ganglia as well as in the cecal content, but in smaller numbers than those detected after 6 h of infection. After IV inoculation (Fig. 1), these bacteria were isolated from Peyer's patches, spleen, liver, and blood during the first 6 h of infection and after 3 days from the spleen and liver, being present in smaller numbers in the liver than during the earlier period. Bacteria were not detected at any of the later times when animals were sacrificed or in the remaining organs, regardless of the route of inoculation.

Strain Yp94, also of serogroup O:3 and without pYV, when inoculated intragastrically (Fig. 2), was found in the cecal material of animals sacrificed 6 h and 3 and 6 days after infection, but in decreasing numbers at each subsequent time. The strain was also isolated from the spleen and liver of animals on the third day of infection. Six hours after IV inoculation (Fig. 2), Yp94 bacteria were isolated from the spleen, liver and blood, and 3 days after inoculation, they were isolated from the spleen in smaller numbers than at the earlier time. Like strain Yp36, strain Yp94 was not isolated from other organs or at any time (6, 10, 15 and 21 days) after the third day.

TABLE
Characteristics of the *Yersinia pseudotuberculosis* strains studied

Strain/bio-serogroups	pYV (kb)	Virulence markers [a]	Origin	Clinical material	Geographical origin	Year
Yp36 (2/O:3)	No pYV	*irp1*, *irp2*	Cattle	Diarrheic feces	Paraná	1989
Yp94 (2/O:3)	No pYV	*irp1*, *irp2*	Buffalo	Normal feces	Rio Grande do Sul	1990
Yp32 (1/O:1a)	79	*lcrF*, *irp1*, *irp2*, *psn*, IS*100*, *ybtE*, *ybtX-ybtS*, *ybtP-ybtQ*	Buffalo	Mesenteric lymph nodes	Rio Grande do Sul	1989
Yp21 (2/O:3)	79	*lcrF*, *irp1*, *irp2*	Swine	Diarrheic feces	Paraná	1985
Yp61 (2/O:3)	71	*lcrF*, *irp1*, *irp2*	Cattle	Diarrheic feces	Paraná	1989

pYV: virulence plasmid *of Yersinia*; *a*: unpublished data, as described by Fukushima et al. (2001); *lcrF*: low calcium response gene; *irp1*: iron-repressible protein 1 gene; *irp2*: iron-repressible protein 2 gene; *psn*: pesticin sensitivity gene ; IS*100*: insertion sequence gene; *ybtE*: yersiniabactin E gene; *ybtX-ybtS*: yersiniabactin X and S genes; *ybtP-ybtQ*: yersiniabactin P and Q genes

Intragastric inoculation

Log $5.3 \times 10^8 = 8.72$

Intravenous inoculation

Log $5.3 \times 10^6 = 6.72$

- ■- Cecal content   - ·□· - Peyer's patches   ···▲··· Mesenteric lymph nodes
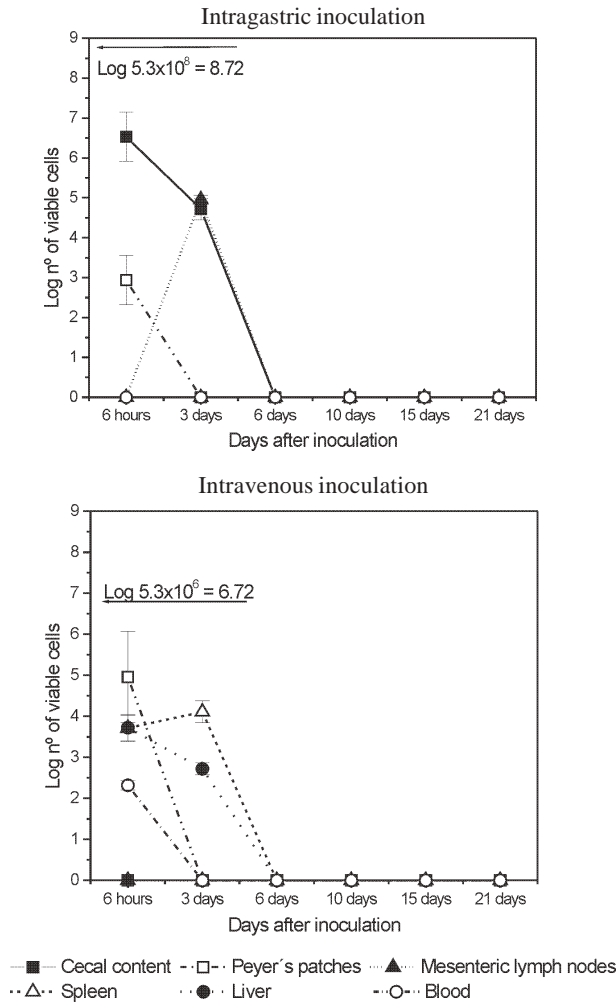- ··△·· Spleen   ·●· Liver   - ··O··· Blood

Fig. 1: experimental infection with strain Yp36: *Yersinia pseudotuberculosis* 2/O:3 not harboring the virulence plasmid and *lcrF* gene, but positive for *irp1* and *irp2* genes, inoculated intragastrically and intravenously into female Swiss mice.

No clinical alterations or macroscopic lesions were observed in mice infected with strains Yp36 or Yp94.

The Yp32 strain of serogroup O:1a, which harbored the pYV plasmid, was the most virulent. After IG inoculation (Fig. 3), the strain was isolated from all organs after 6 h of infection, being undetectable only in the blood. The animals developed diarrhea between the 3rd and 6th day of infection and those sacrificed on the 6th day presented enlarged liver and spleen. Although they recovered from diarrhea after the 10th day of infection, four of them died during the experiment. After IV infection (Fig. 3), the experiment could only be continued to the 10th day, when the five surviving animals were sacrificed, the rest of the group of 35 having died before the 10th day. After 6 h, as well as after 3 and 6 days of infection, bacteria had invaded all organs and tissues examined and were able to multiply in them. Clinical examination of the animals showed severe diarrhea with lesions in the various organs and increased Peyer's patch volume and enlarge-

ment of the mesenteric ganglion chain.

After IG infection (Fig. 4), strain Yp21 of serogroup O:3, which harbors the pYV plasmid, was isolated from various organs up to the 15th day of infection. The animals did not suffer diarrhea, but were apathetic during the 1st days after infection, and four of them died. Those sacrificed on the 3rd day exhibited lesions in the liver. This strain showed greater aggressiveness after IV infection (Fig. 4), since no animal survived until the 21st day. The experiment was therefore finished on the 15th day, when all surviving animals were sacrificed. The bacterium infected all organs examined on the 3rd and 6th days after infection. After 6 h, it was isolated from the spleen, liver and blood, and on the 15th day it was isolated from cecal content and Peyer's patches. The animals developed diarrhea, macroscopic lesions appeared in the liver and spleen and the volume of Peyer's patches increased.

When inoculated by the IG route (Fig. 5), the Yp61 strain of serogroup O:3, which also carried the pYV plas-

Intragastric inoculation

Log $2.9 \times 10^8 = 8.46$

Intravenous inoculation

Log $2.9 \times 10^6 = 6.46$

- ■- Cecal content   - ·□· - Peyer's patches   ···▲··· Mesenteric lymph nodes
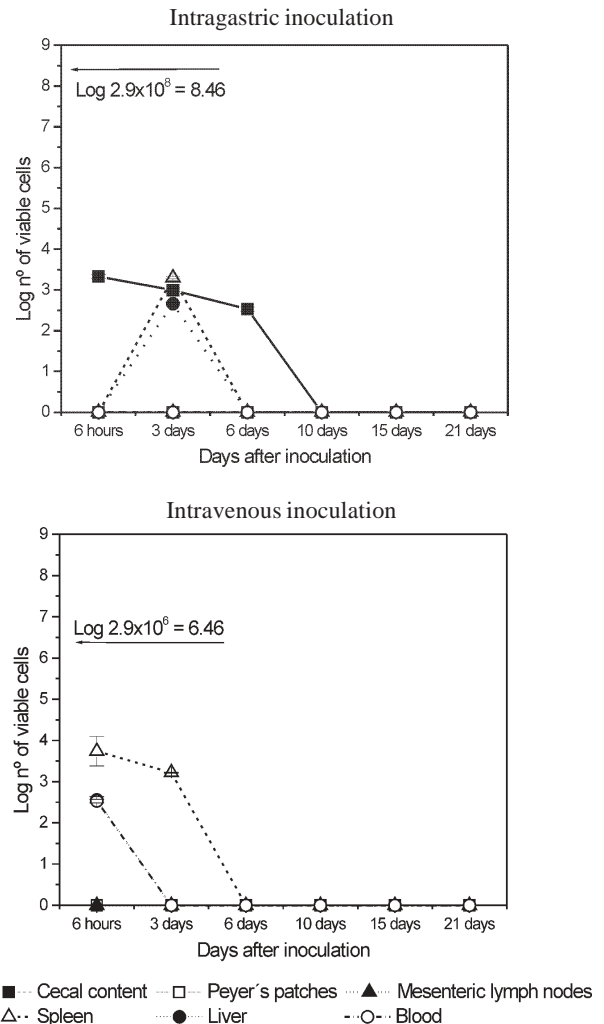- ··△·· Spleen   ·●· Liver   - ··O··· Blood

Fig. 2: experimental infection with strain Yp94: *Yersinia pseudotuberculosis* 2/O:3 not harboring the virulence plasmid and *lcrF* gene, but positive for *irp1* and *irp2* genes, inoculated intragastrically and intravenously into female Swiss mice.

Intragastric inoculation

$Log\ 3 \times 10^8 = 8.48$

Intragastric inoculation

$Log\ 6.4 \times 10^8 = 8.81$

Intravenous inoculation

$Log\ 3 \times 10^6 = 6.48$

Intravenous inoculation

$Log\ 6.4 \times 10^6 = 6.81$

—■— Cecal content - -□- - Peyer's patches ·······▲······· Mesenteric lymph nodes
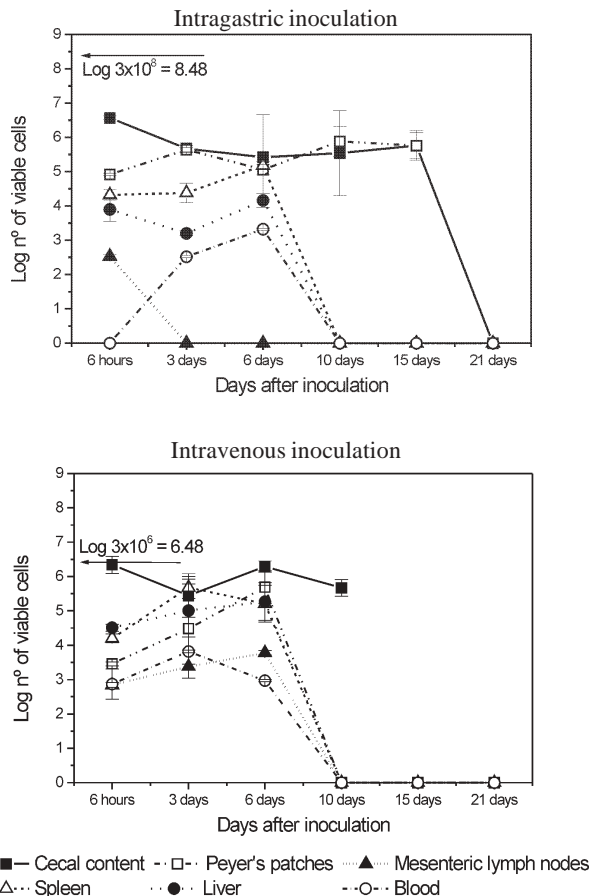···△··· Spleen   · ·●· · Liver   - -○- - Blood

Fig. 3: experimental infection with strain Yp32: *Yersinia pseudotuberculosis* 1/O:1a harboring the virulence plasmid and positive for *lcrF*, *irp1*, *irp2*, *psn*, IS*100*, *ybtE*, *ybtX-ybtS*, and *ybtP-ybtQ* genes, inoculated intragastrically and intravenously into female Swiss mice.
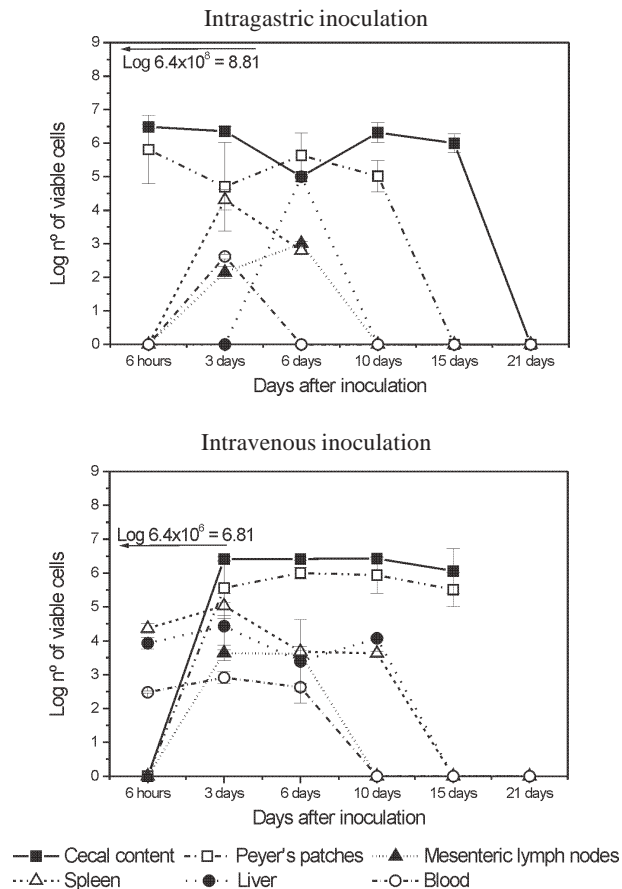
Fig. 4: experimental infection with strain Yp21: *Yersinia pseudotuberculosis* 2/O:3 harboring the virulence plasmid and positive for *lcrF*, *irp1,* and *irp2* genes, inoculated intragastrically and intravenously into female Swiss mice.

mid, was not isolated either 6 h after inoculation or on the 21st day of infection from any of the sites examined. It caused prostration in animals, but not diarrhea. Macroscopic examination of the spleen, liver and Peyer's patches of the animals sacrificed on the 6th day showed enlargement of these organs and five of them died during the experiment. Six hours after IV inoculation (Fig. 5), bacteria were found only in the spleen, liver and blood, while after 15 days they were found in cecal content, Peyer's patches, and the mesenteric lymph nodes. The experiments were concluded on the 15th day when the five surviving animals were sacrificed, all other animals having died between the 6th and 10th day after infection. After IV inoculation, the animals presented diarrhea and emaciation, apathy and ruffled hair, and enlarged spleen and liver, with macroscopic lesions appearing between the 3rd and 6th day.

DISCUSSION

The results presented in this study show that the microorganism's ability to invade and multiply in the infected animals is directly related to the presence of the pYV plasmid, regardless of the inoculation route.

The ability of *Y. pseudotuberculosis* to neutralize the effects of the host's immune system and thus survive and spread through the spleen and liver depends on whether it harbors this plasmid. The genes carried on pYV express components of the type-III secretion system, including effector Yops molecules (Cornelis et al. 1998). Some investigators (Holmström et al. 1995, Monack et al. 1998) have demonstrated that Yops are responsible for the ability of *Yersinia* to multiply in Peyer's patches, to invade tissues, and to cause the death of infected mice. However, by means of electron microscopy, Simonet et al. (1990) found evidence that *Y. pseudotuberculosis* strains that lose the pYV plasmid are able to multiply during the early stages of infection in a similar fashion to that of strains harboring the plasmid, probably through the action of the chromosomal virulence factors present.

Two of the five *Y. pseudotuberculosis* strains used in this study did not harbor the plasmid, although one of these was isolated from a sick animal (Yp36) and the other from a healthy animal (Yp94). Both showed quite similar infection kinetics, not being very invasive and practically not multiplying in the organs of the animals, regardless of the route of infection.

Intragastric inoculation

Intravenous inoculation

—■— Cecal content   ··□·· Peyer´s patches   ···▲··· Mesenteric lymph nodes
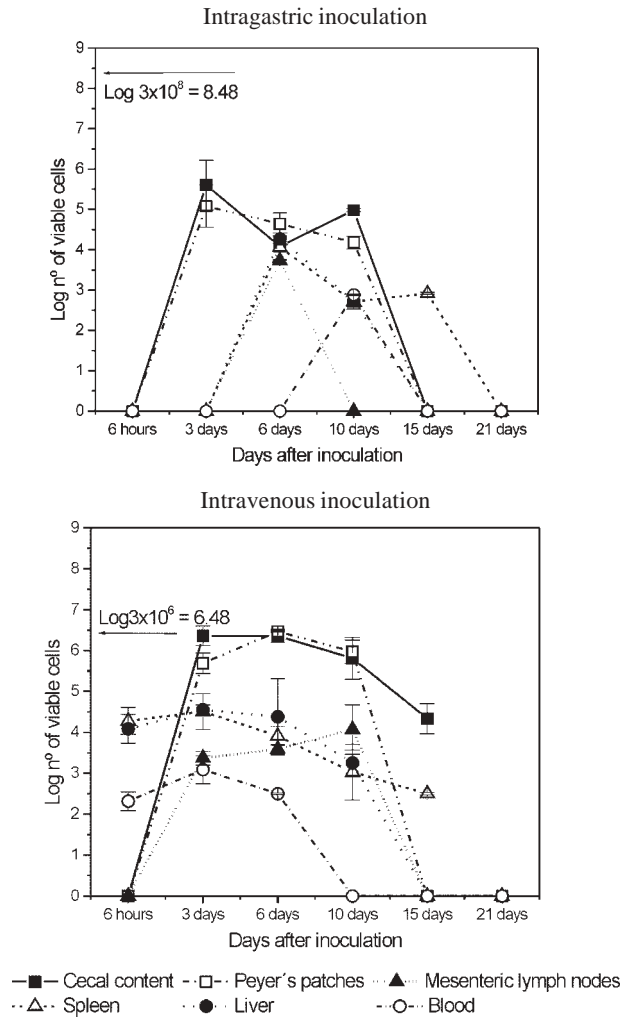··△·· Spleen   ··●·· Liver   ··○·· Blood

Fig. 5: experimental infection with strain Yp61: *Yersinia pseudotuberculosis* 2/O:3 harboring the virulence plasmid and positive for *lcrF*, *irp1*, and *irp2* genes, inoculated intragastrically and intravenously into female Swiss mice.

It should be pointed out that the animals inoculated with strains Yp36 and Yp94, which did not harbor the virulence plasmid, did not show any clinical alterations or any macroscopic lesions in any of the organs examined during the 21 days of the experiment.

The presence of the pYV plasmid in the other strains studied (Yp32, Yp21, and Yp61) caused the infection to develop differently from that caused by plasmidless strains, Yp36 and Yp94, since the mice suffered more severe infection.

The evolution data for in vivo infection showed greatest aggressiveness in the Yp32 strain belonging to serogroup O:1a. This strain induced diarrhea regardless of the route of inoculation and killed all but five of the animals by the 10th day after IV inoculation. This strain presents a complete high-pathogenicity island (HPI), which is related to the strategy of acquiring iron molecules. It is well known that for bacterial pathogens, the successful establishment of disease depends on the ability of the organisms to acquire iron. In *Yersinia* one of the major differences between strains with low and high pathogenity lies in their ability to capture iron molecules in vivo and thus to disseminate and cause systemic infections (Carniel 1999).

The only strain that presents all genes of HPI was the Yp32. Also it was the only strain able to infect all sites when examined 6 hours after IV inoculation, and all except blood, 6 hours after IG infection. These results are in agreement with those in the literature, since *Y. pseudotuberculosis* O:1a strains are classified as highly virulent (Rakin et al. 1995, Monack et al. 1998).

The other two strains (Yp61 and Yp21), although harboring the virulence plasmid, were less virulent than Yp32, probably because they did not possess a complete HPI. They caused diarrhea after IV but not after IG inoculation. Similar data are reported by Cornelis et al. (1987) in relation to *Y. enterocolitica*; according to them, diarrhea or loose stools are not common features of yersiniosis in mice infected intragastrically with the *Y. enterocolitica* O:8 (American strain), a situation that reflects the human infection, in which fever and abdominal pain without diarrhea are the common clinical manifestations of the disease.

It is very well known that the LD_{50} for *Y. pestis* and virulent strains of *Y. enterocolitica* and *Y. pseudotuberculosis* is very low for some types of mice, but not for others. Several studies have highlighted differences in the resistance of various mouse strains to intravenous infection with *Y. enterocolitica*. (Hancock et al. 1986, Heesemann et al. 1993, Autenrieth et al. 1994). By the other hand, Handley et al. (2004) working with different strains of inbred mice related that the animals presented no differences in 50% lethal dose (LD_{50}) following oral infection with *Y. enterocolitica*.

It is important to emphasize that in the present work with *Y. pseudotuberculosis,* the animal model used was female Swiss mice aged 5 to 6 weeks. This model has been used in previous studies to determine the kinetics of infection of various species of *Yersinia*, including the highly pathogenic *Y. enterocolitica* O:8 WA; also the same routes of infection (IG and IV) were used, as well as similar doses: 10^6 cells/ml for IV and 10^8 cells/ml for IG inoculation (Falcão et al. 1984, Bauab & Falcão 1991).

The present results also confirm the interdependence between virulence markers located on the pYV plasmid and on the chromosome for the full expression of *Y. pseudotuberculosis* disease, as previously reported by Carniel (2001) and Fukushima et al. (2001).

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