

# Shiga toxin-producing *Escherichia coli* O157:H7 shows an increased pathogenicity in mice after the passage through the gastrointestinal tract of the same host

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Haemolytic uraemic syndrome (HUS) is a rare but life-threatening complication of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) infections, characterized by acute renal failure, thrombocytopenia and haemolytic anaemia. Although the main infection route is the consumption of contaminated food or water, person-to-person transmission has been suggested in several situations. Moreover, epidemiological data indicate that the horizontal transmission of several pathogens, including STEC, among individuals of the same species requires significantly lower doses than those used in animal models infected with laboratory-cultured bacteria. Thus, the aim of this study was to evaluate whether the passage of a clinically isolated STEC strain through the gastrointestinal tract of mice affects its pathogenicity in mice. To test this, weaned mice were orally inoculated by gavage with either an *E. coli* O157:H7 isolate from an HUS patient, or the same strain recovered from stools after one or two successive passages through the gastrointestinal tract of the mice. We show that stool-recovered strains are able to induce a more generalized and persistent colonization than the parent strain. Furthermore, a 10<sup>4</sup>-fold-reduced inoculum of the stool-recovered strains still causes gut colonization and mouse mortality, which are not observed with the parent strain. These results indicate an increased pathogenicity in stool-recovered strains that may be associated with an increased ability to colonize the mouse intestine.

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## INTRODUCTION

Haemolytic uraemic syndrome (HUS) is characterized by the triad microangiopathic haemolytic anaemia, thrombocytopenia and nephropathy (Gianantonio *et al.*, 1973). Shiga toxin (Stx)-producing *Escherichia coli* (STEC) O157:H7 has been recognized as the most frequent serotype associated with large outbreaks or sporadic cases of haemorrhagic colitis and HUS in many countries (Griffin & Tauxe, 1991). Although the main virulence factor of STEC is the production of one or more types of Stx (Stx1, Stx2 or their variants), adherence to the intestinal epithelium and gut colonization are also important components of the pathogenesis. Moreover, the degree of gut adhesion has been correlated with the ability to cause disease (Frankel *et al.*,

1998; Turner *et al.*, 2006). In humans, STEC infection and HUS usually occur after the consumption of contaminated food or water, having a minimal infective dose of <10 bacteria per gram of food (Paton *et al.*, 1996). Several attempts have been made to develop reliable animal models of STEC infection and HUS pathogenesis. However, in order to establish infection, the use of very large doses of viable micro-organisms is needed since STEC strains are poorly pathogenic in other animal species. Furthermore, some HUS animal models rely on the manipulation of the intestinal environment with different drugs such as antibiotics (Lindgren *et al.*, 1993; Wadolowski *et al.*, 1990) or mitomycin C (Fujii *et al.*, 1994) in order to establish reproducible infections. However, these treatments could disturb the immune response of the host (Domingo *et al.*, 1995), alter Stx excretion (Grif *et al.*, 1998) or induce microangiopathy (Molyneux *et al.*, 2005).

Abbreviations: HUS, haemolytic uraemic syndrome; STEC, Shiga toxin-producing *Escherichia coli*; Stx, Shiga toxin.

On the other hand, epidemiological data on several infectious agents indicate that horizontal transmission among animals of the same species requires significantly lower infective doses than those used in animal models (Merrell *et al.*, 2002; Wiles *et al.*, 2005). In this regard, previous reports have demonstrated that calves have a high prevalence of natural infection when they are exposed to low doses of *E. coli* O157:H7 within a herd (Besser *et al.*, 2001; Cray & Moon, 1995; Garber *et al.*, 1995; Hancock *et al.*, 1997). Similarly, donor pigs that shed very low levels of *E. coli* O157:H7 in their faeces are demonstrated to transmit these bacteria to naïve pigs that shared the same herd (Cornick & Helgerson, 2004). This effect has also been reported in the natural transmission of *Salmonella* Typhimurium (Fedorka-Cray *et al.*, 1994) and *Salmonella* Choleraesuis (Gray *et al.*, 1996) in swine, and *Citrobacter rodentium* in mice (Wiles *et al.*, 2005), after exposure to low levels of bacteria in the environment. Although this fact has not yet been fully explained, it probably reflects the importance of bacterial–host interaction in the infection establishment by inducing bacterial pathogenicity or in the maintenance of an animal reservoir. Additionally, person-to-person transmission in HUS cases has been proposed in several circumstances (Bell *et al.*, 1994; Crump *et al.*, 2002; Karmali, 1989). Thus, the aim of this study was to evaluate whether the passage of a human-isolated STEC strain through the gastrointestinal tract of the mouse affects its pathogenicity in mice.

We have previously developed an HUS mouse model in weaned BALB/c mouse by oral administration of an *E. coli* O157:H7 isolate from an HUS patient (Brando *et al.*, 2008). In that work we found that the bacterial administration by gavage causes a transient intestinal colonization and symptoms of Stx2-mediated systemic toxicity in a percentage of the inoculated mice. Herein, we studied the influence of successive passages through the gastrointestinal tract of the mouse on the pathogenicity of STEC strains by using the same HUS mouse model. We demonstrate that stool-recovered strains are able to generate a more generalized and persistent colonization than the parent strain. Furthermore, we show that the use of a significantly lower inoculum of the stool-recovered strains ( $10^4$ -fold-reduced) still results in gut colonization and mouse mortality. These results suggest that stool-recovered strains have a higher pathogenic capacity even though Stx2 production was not increased.

Our results highlight the importance of bacterial adaptation to the host gut environment for the colonization process and the establishment of STEC infections. Although the molecular mechanisms involved in these processes remain to be elucidated, our experimental model represents a new approach for using animal models to study STEC infections.

## METHODS

**Mice and oral infection.** Immature male and female BALB/c mice were used immediately after weaning (17–20 days of age, ~8–11 g of body weight). BALB/c mice were bred in the animal facility at the

Department of Experimental Medicine, Academia Nacional de Medicina, Buenos Aires. Experiments performed herein were approved by the Academia Nacional de Medicina Animal Care Committee. Weaned mice were divided randomly into experimental groups. After 8 h of starvation (Fernandez *et al.*, 2003; Nagano *et al.*, 2003), animals were intragastrically inoculated via a stainless steel canula (model 7.7.1, 0.38 mm x 22G) (Harvard Apparatus) with 0.1 ml of the corresponding bacterial suspension. Four hours after the ingestion of the bacterial suspension, food and water were provided to mice *ad libitum*. Animals were observed daily for activity level and water intake for up to 10 days, when survivors were euthanized. Blood and stool samples were examined at 48, 72 and 96 h after infection.

**Bacterial strains.** Bacterial strains used in this study included a strain isolated from a faecal specimen of a child with HUS (125/99), (previously characterized in Brando *et al.*, 2008) and two strains recovered from faecal samples of inoculated mice (125r and 125rr), which were obtained as follows: mice at weaning were intragastrically inoculated with a total inoculum of the 125/99 strain (parent strain) at a dose of  $6 \times 10^9$  c.f.u.  $\text{kg}^{-1}$ . Forty eight hours later, stool samples were obtained and cultured onto Sorbitol MacConkey agar plates. One non-sorbitol-fermenting colony was isolated and characterized by the presence of *stx2/rfbO157* with a multiplex PCR (Leotta *et al.*, 2005) and serotyped with somatic and flagellar antisera from the Instituto Nacional de Producción de Biológicos- ANLIS 'Dr Carlos G. Malbrán' (Orskov & Orskov, 1984). The reference *E. coli* strains EDL933 O157:H7 (*stx1* and *stx2*), and ATCC 25922 were used as positive and negative controls of gene expression, respectively. This strain was named 125r, and after culturing in tryptic soy broth (TSB; Difco), it was intragastrically inoculated into weaned mice at a dose of  $2 \times 10^8$  c.f.u.  $\text{kg}^{-1}$ . One non-sorbitol fermenting colony was isolated from stools of inoculated mice and characterized by PCR and serotyped as described previously. This strain, isolated from mouse stools after two consecutive passages through the gastrointestinal tract of a mouse, was named 125rr and, after culturing in TSB, was intragastrically inoculated in weaned mice at a dose of  $2 \times 10^8$  c.f.u.  $\text{kg}^{-1}$ . Both strains, the 125r and 125rr, were collectively named stool-recovered strains, and were maintained at  $-70^\circ\text{C}$  in TSB supplemented with 20% (v/v) glycerol.

**Bacterial growth.** Bacterial culture was performed as described previously (Brando *et al.*, 2008). Briefly, strains were cultured overnight at  $37^\circ\text{C}$  in TSB. A 250  $\mu\text{l}$  volume of each strain was inoculated into five Erlenmeyer flasks (125 ml) containing 25 ml TSB and incubated at  $37^\circ\text{C}$  for 18 h. Cultures were centrifuged and bacterial pellets were washed twice in PBS (pH 7.2; 0.01 M) and then resuspended in 1 ml of PBS. Aliquots were diluted ( $10^2$ – $10^4$ ), plated onto Plate Count Agar, and incubated overnight at  $37^\circ\text{C}$ . Then, c.f.u.  $\text{ml}^{-1}$  was determined. Overnight cultures reached a final concentration of  $1 \times 10^{10}$ – $1.8 \times 10^{10}$  c.f.u.  $\text{ml}^{-1}$ .

**Pulsed-field gel electrophoresis (PFGE).** Macrorestriction fragments analysis by PFGE was performed using the 24 h PulseNet standardized PFGE protocol for *E. coli* O157:H7 (Ribot *et al.*, 2006). The PulseNet strain used as a size standard was *Salmonella* Braenderup H9812 (kindly provided by the Center for Disease Control, Atlanta, GA, USA). Restriction digestion of DNA was carried out with *XbaI* enzyme (Promega). PFGE images of gels were obtained by Doc-It 2000 (Bio-Rad). Analysis of TIFF images was carried out through the BioNumerics version 4.61 software package (Applied Maths) by using the Dice coefficient and the unweighted pair group method with arithmetic mean (UPGMA) to generate dendrograms with 1.5% tolerance values. Clusters were visually confirmed.

**In vitro quantification of Shiga toxin production.** Bacterial supernatants of each strain were tested for Stx2 production by a

standardized ELISA method according to manufacturer's indications (Ridascreen Verotoxin, R-Biopharm AG). In order to quantify the Stx2, a standard curve was performed using serial dilutions of purified Stx2 (Phoenix Lab, Tufts Medical Center, Boston, MA, USA). Then, absorbance values of each strain were interpolated into the dose-response curve and the Stx2 concentration was determined. The total Stx2 production was calculated as the toxin released in the culture supernatant plus the fraction stored in the periplasmic compartment and released into the supernatant after polymyxin B treatment (Karmali, 1989; Karmali *et al.*, 1985). Each bacterial supernatant was evaluated in duplicate in two independent cultures.

**Colonization: isolation and enumeration of STEC strains from mouse intestine.** Mice were killed at 72 and 96 h after bacterial inoculation to determine intestinal colonization. Small and large intestines were excised and stools were removed and diluted to 0.1 g ml<sup>-1</sup>. Each part of the intestine (5 cm each) was washed three times vigorously with 6 ml of PBS by using biopsy forceps. Then, each intestinal section was homogenized in 0.5 ml PBS. Viable counts were performed by plating 100 µl stool samples or homogenized intestinal tissues onto SMAC agar and incubating overnight at 37 °C. Non-sorbitol-fermenting colonies were counted and selected at random for confirmation by serotyping and multiplex PCR as described previously. The number of c.f.u. per intestinal section was calculated by considering the c.f.u. ml<sup>-1</sup> and the total volume of each sample.

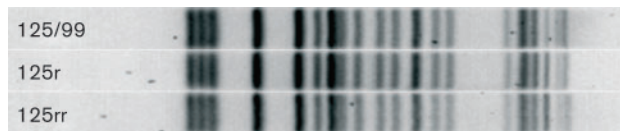
**Haematological studies.** Blood samples were obtained by puncturing the retroorbital plexus at 48, 72 and 96 h after bacterial feeding for laboratory analyses that included total and differential blood cell counts in a Neubauer chamber, blood smears and blood urea nitrogen determination. Biochemical determination of urea content in mouse plasma was performed in an autoanalyser CCX spectrum (Abbot Diagnostics Systems), following standardized instructions. Values higher than the mean ± 2SD of age-matched normal mice were considered as increased.

**Statistics.** Survival data were analysed for significance by using log-rank tests. Data expressed as the mean ± SEM of individual mice were analysed for statistical differences by using one- or two-way analysis of variance (ANOVA). Comparisons, *a posteriori*, between two groups were performed using the Student–Newman–Keuls (SNK) test. Median values were compared using the Wilcoxon Signed Rank test.

## RESULTS

### Subtyping of bacterial strains

In order to characterize and establish the identity of the stool-recovered strains with the parent strain, the macro-restriction patterns of the DNA fragments generated by the *Xba*I enzyme were compared as described in Methods. Fig. 1 shows that the *Xba*I-PFGE profiles from the three strains were indistinguishable, thus indicating that the stool-recovered strains had the same clonal origin as the parent strain. We also confirmed that all strains had the same serotype (O157:H7) and genotype (*eae/ehxA/stx2*) determined as described in Methods. However, Stx2 production was significantly decreased in both stool-recovered strains compared to the parent strain as quantified by an ELISA [mean Stx2 production ± SEM (µg Stx2 µl<sup>-1</sup>) = 125/99, 491 ± 102; 125r, 298 ± 63; 125rr, 274 ± 22; two-way ANOVA *P* < 0.001. Similar results were obtained in the



**Fig. 1.** *Xba*I profile of the studied strains. Dendrograms were obtained with 1.5% of tolerance and compared by the DICE coefficient as described in Methods. The *Xba*I-PFGE pattern obtained for the different strains was the same (AREXHX01-0011).

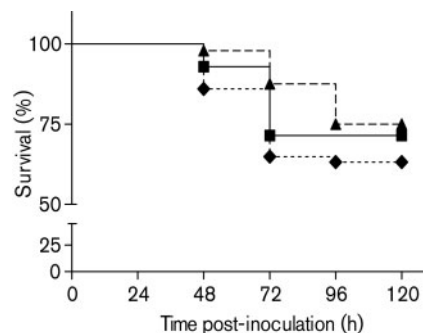
evaluation of Stx2 biological activity on VERO cell cultures (data not shown).

### Effect of strain inoculation on mouse survival

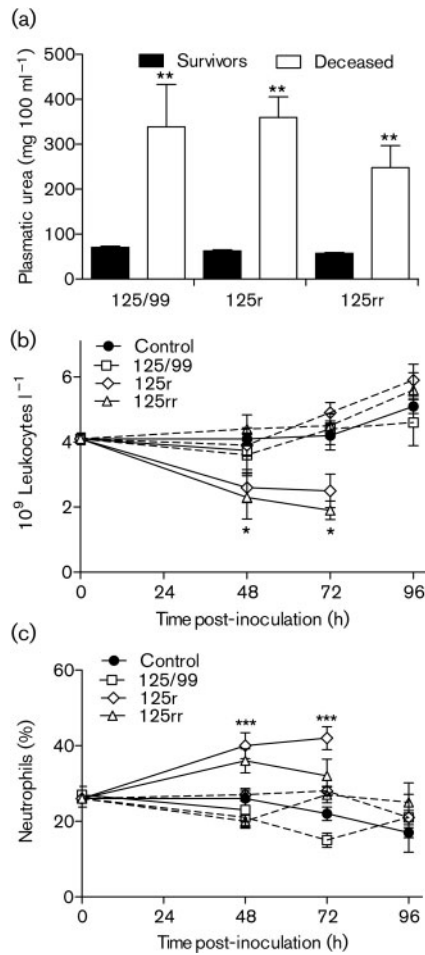
Since mortality within 24 h post-inoculation was considered to be caused by endotoxic shock, the bacterial dose was adjusted to minimize early deaths. Thus,  $2 \times 10^8$  c.f.u. kg<sup>-1</sup> each strain was administered by gavage to BALB/c mice at weaning. Mice were observed daily, and blood and stool samples were collected until death. Differences were not observed in the survival rates at this bacterial dose in spite of the lower Stx2 production by the stool-recovered strains (Fig. 2).

### Clinical evaluation of infected mice

To assess the systemic effect of bacterial infection, blood samples were obtained daily. Plasmatic urea levels (Fig. 3a) and total (Fig. 3b) and differential (Fig. 3c) leukocyte counts were evaluated. Data were retrospectively analysed according to the final outcome of the inoculated mice. Mice that died showed increased urea levels compared to the survivors at 72 h after inoculation (Fig. 3a). On the

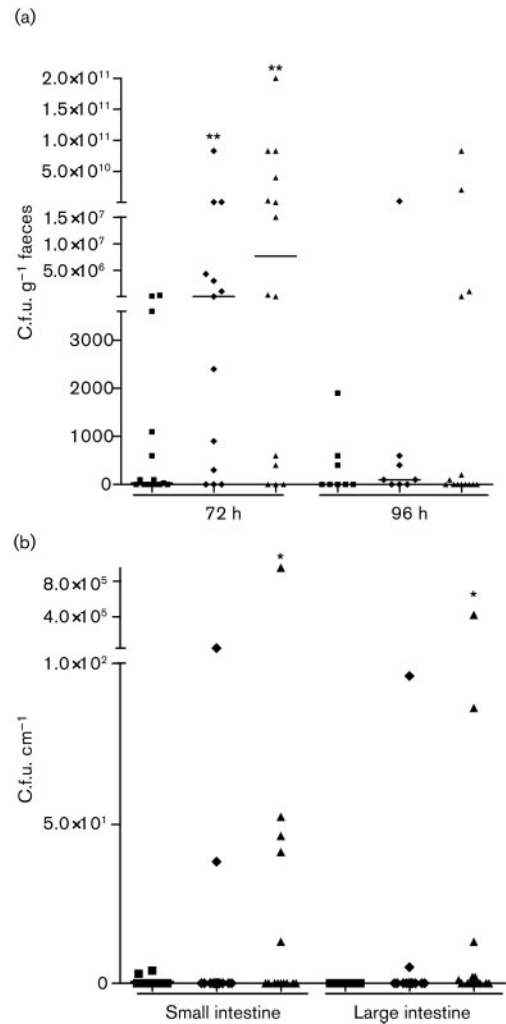


**Fig. 2.** Survival rates of mice after bacterial inoculation. 17–19 day-old mice were orally inoculated with  $2 \times 10^8$  c.f.u. kg<sup>-1</sup> of the indicated STEC strain (■, 125/99; ◆, 125r; and ▲, 125rr). Survival of mice was monitored every 6 h after bacterial inoculation. Survival percentages corresponding to at least four pooled experiments with at least five mice per group are shown (*n* = 20–63 mice by bacterial strain).



**Fig. 3.** Clinical parameters in inoculated mice. Mean  $\pm$  SEM of plasmatic urea values corresponding to at least four pooled experiments with 4–36 mice per group is shown. Mice were classified retrospectively in two groups according to their final outcome (survivors or deceased). Data were compared by ANOVA multiple comparison test and *a posteriori* by SNK test. (a) Plasmatic urea levels at 72 h after bacterial inoculation. ANOVA,  $**P < 0.0001$ ; SNK,  $P < 0.0001$  compared with survivors. (b, c) Peripheral white cell counts after bacterial inoculation. Mice were bled at 48, 72 and 96 h after oral inoculation (broken lines represent survivors and solid lines represent deceased). (b) Total leukocyte counts. ANOVA,  $*P < 0.05$ ; 72 h SNK,  $P < 0.05$ ; strains 125r and 125rr deceased compared to survivors. (c) Percentage of neutrophils. ANOVA,  $***P < 0.0001$ ; 48 h SNK,  $P < 0.05$ ; strains 125r deceased compared to 125/99 deceased and survivors and 125rr deceased compared to 125/99 and 125rr survivors; 72 h SNK,  $P < 0.001$  125r deceased compared to survivors  $P < 0.05$  125rr deceased compared to 125r deceased and 125/99 and 125r survivors.

other hand, only those mice that died after the inoculation of stool-recovered strains showed a significant increase in the percentage of circulating neutrophils (Fig. 3c) with a significant decrease in total circulating leukocytes (Fig. 3b) since 48 h after bacterial administration. Survivor mice



**Fig. 4.** Bacterial isolation from stools (a) and small and large intestine (b) from inoculated mice. Mice were euthanized at 72 and 96 h after intragastric inoculation of 125/99 (■), 125r (◆) and 125rr (▲) bacterial strains and the number of sorbitol-negative c.f.u. was determined in stools (72 and 96 h) and intestines (72 h) as described in Methods. Each symbol represents an individual mouse from four pooled experiments and solid lines represent median values. The detection limit in stools was 200 c.f.u.  $g^{-1}$  and in small and large intestine was 1 c.f.u.  $cm^{-1}$ . (a)  $**P < 0.01$  compared to 125/99 at the same time (b)  $*P < 0.05$  compared to 125/99.

from all experimental groups showed leukocyte counts similar to the control group.

### Colonization of intestinal epithelium

The number of bacteria shed in stools (Fig. 4a and Table 1) and adhered to small and large intestines (Fig. 4b and Table 1) was quantified at 72 and 96 h after inoculation. All samples with 200 c.f.u.  $g^{-1}$  faeces or more were considered positive. The group of mice inoculated with stool-



**Table 1.** Bacterial isolation from faeces and intestinal tissues at 72 h after inoculation

Rank, number of c.f.u. isolated in positive samples; Ratio, no. of mice with at least one STEC-positive culture/total no. of mice evaluated; %, percentage of mice with positive samples.

Strain	Faeces				Small intestine			Large intestine		
	Median	Rank (c.f.u. g <sup>-1</sup> )	Ratio	%	Rank (c.f.u. cm <sup>-1</sup> )	Ratio	%	Rank (c.f.u. cm <sup>-1</sup> )	Ratio	%
125/99	3 × 10 <sup>1</sup>	6 × 10 <sup>2</sup> –2 × 10 <sup>5</sup>	5/15	33	3–4	2/14	14	0	0/14	0
125r	1 × 10 <sup>4</sup> *	3 × 10 <sup>2</sup> –8 × 10 <sup>10</sup>	10/13	77†	4 × 10 <sup>1</sup> –1 × 10 <sup>4</sup>	2/13	15	5–1 × 10 <sup>2</sup>	2/13	15
125rr	8 × 10 <sup>6</sup> *	4 × 10 <sup>2</sup> –2 × 10 <sup>11</sup>	11/14	79†	1 × 10 <sup>1</sup> –1 × 10 <sup>6</sup>	5/14	36	1–4 × 10 <sup>5</sup>	6/14	43†

\**P*<0.001 compared to 125/99

†*P*<0.05 compared to 125/99.

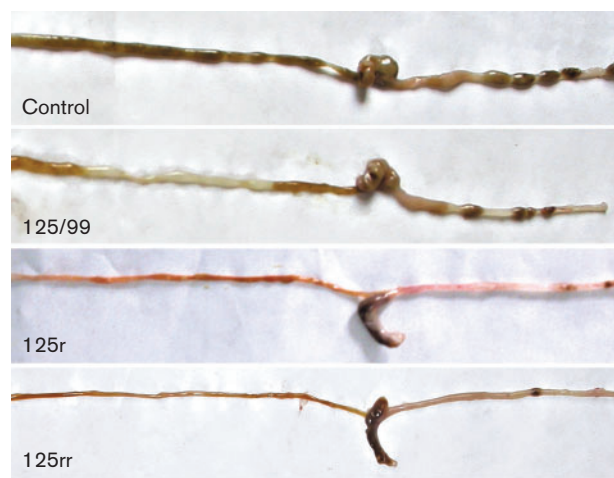
recovered strains had a significantly higher ratio of mice with positive isolates and higher numbers of c.f.u. g<sup>-1</sup> in faeces samples than the group of mice inoculated with the parent strain (Fig. 4a and Table 1). In addition, the group of mice inoculated with the 125rr strain showed a higher ratio of STEC-positive isolates from the large intestine as well as a higher number of c.f.u. recovered from both intestinal segments than the parent strain at 72 h (Fig. 4b and Table 1). On the other hand, the parent strain was scarcely recovered from small intestine. These results demonstrate that stool-recovered strains were able to generate a more generalized and persistent colonization in the intestine of weaned mice than the parent strain. Additionally, the 125rr strain showed the highest adherence to both intestinal segments. To confirm that counted colonies were indeed STEC, randomly selected non-sorbitol-fermenting colonies were examined for the presence of *stx1*, *stx2* and *rfbO157* genes by multiplex PCR. All tested colonies were positive for *rfbO157* and *stx2* genes (data not shown).

### Macroscopic examination of the intestine of inoculated mice

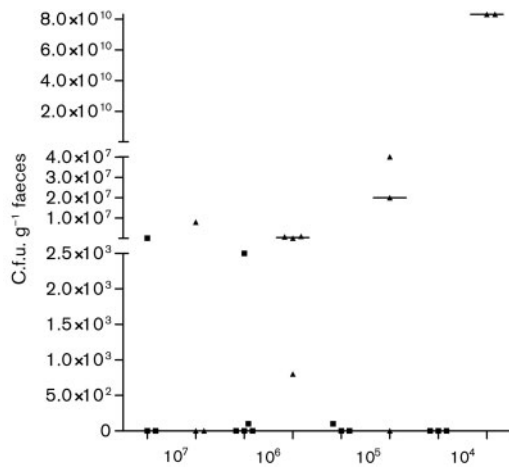
In order to establish whether the more generalized colonization observed in the animals inoculated with the stool-recovered strains leads to the development of differential histological alterations, intestines of mice were examined macroscopically. Fig. 5 shows a representative animal from each experimental group. Although small intestines from animals inoculated with all bacterial strains showed macroscopic alterations, those from animals inoculated with stool-recovered strains showed the most widely distributed range of alterations, i.e. they were sticky, considerably empty, flimsy, breakable, and noticeably irrigated. On the other hand, the distal colon from animals inoculated with the parent strain contained formed stool pellets, whereas the proximal colon contained semisolid stools. In contrast, large intestines from animals inoculated with stool-recovered strains were devoid of content and noticeably irrigated.

### Pathogenicity at suboptimal inocula

To study whether the higher ability of the stool-recovered strains to colonize the gut leads to an increased pathogenicity in mice, several lower doses of the parent or 125rr strains were intragastrically administered to mice at weaning. The number of c.f.u. g<sup>-1</sup> of faeces at 72 h after inoculation and the survival rate were evaluated. Mice inoculated with 125rr strain at doses of 10<sup>7</sup>, 10<sup>6</sup>, 10<sup>5</sup> and even 10<sup>4</sup> c.f.u. kg<sup>-1</sup> showed shedding of bacteria in faeces at 72 h (Fig. 6). This strain was also able to induce 46 and 14% mortality at doses of 1 × 10<sup>7</sup> and 1 × 10<sup>4</sup> c.f.u. kg<sup>-1</sup>, respectively (data not shown). In contrast, the parent strain was scarcely isolated from faeces and did not induce death in mice inoculated with these dosages.



**Fig. 5.** Macroscopic examination of the intestines. Whole intestines from mice inoculated with PBS, the parent STEC strain (125/99) and stool-recovered STEC strains (125r and 125rr) at 72 h. One representative intestine of each group is shown. Pictures were taken with a Nikon Coolpix 4500 digital camera.



**Fig. 6.** Bacterial isolation from faeces after suboptimal inocula. Mice were euthanized at 72 h after intragastric inoculation of 125/99 (■) and 125rr (▲) bacterial strains and the number of sorbitol-negative c.f.u. was determined in stools as described in Methods. Each symbol represents an individual mouse from two pooled experiments and solid lines represent the median values.

## DISCUSSION

One of the difficulties encountered in the establishment of a mouse model of HUS by gastric infection is the fact that STEC strains are not pathogenic in mice. Thus, the development of a reliable and reproducible HUS model requires large infective doses. In this study, we show that a human-isolated STEC strain is able to generate a more generalized and persistent colonization of the mouse intestine after the passage through the gastrointestinal tract of the same species, thus providing a useful tool to investigate colonization and pathology in mice.

Stool-recovered strains showed both faecal shedding and an adherence to intestinal tissue that was significantly increased compared to the parent strain when inoculated into weaned mice. Moreover, the group of mice inoculated with the 125rr strain, which had two successive passages through the gastrointestinal tract of mice, exhibited the highest ratio of mice with STEC-positive isolates from the large intestine and the highest number of c.f.u. per gram of faeces and per centimetre of tissue. These findings suggest that stool-recovered strains display additional advantages in the colonization process. It has been suggested that once pathogens adhere to and colonize the intestinal epithelia, they sense microenvironmental conditions such as temperature, nutrient availability and presence of stress hormones, thus adjusting their transcriptome/proteome accordingly (Sperandio *et al.*, 2003). Moreover, the study of the transcriptional profile of bacteria directly isolated from faeces revealed a unique behaviour characterized by the high expression of genes required for nutrient acquisition and motility (Merrell *et al.*, 2002). In a similar way, a uropathogenic *E. coli* strain isolated from the urine

of experimentally infected mice showed upregulation of genes encoding adherence factors, drug resistance proteins, antimicrobial peptides and proteins involved in iron acquisition systems and the uptake and catabolism of different sources of carbon (Snyder *et al.*, 2004). In our model, even though stool-recovered strains were grown in TSB after isolation and before mouse administration, they still showed an increased ability to colonize the mouse intestine. Also, a second successive passage through the gastrointestinal tract of mice further increased their ability to colonize the mouse intestine.

Intestines from mice inoculated with stool-recovered strains revealed larger macroscopic alterations than those from mice fed with the parent strain. They appeared particularly sticky, considerably empty, flimsy, breakable and noticeably irrigated. In addition, stool-recovered strains showed a differential colonization pattern, since they were isolated from the large intestine of inoculated mice. Previous reports have demonstrated that the horizontal transmission of certain bacteria among animals of the same species changes the infection pattern in comparison to that observed in experimental infections (Besser *et al.*, 2001; Dekker *et al.*, 1995; Gray *et al.*, 1996; Linton *et al.*, 1985; Proux *et al.*, 2001; Shere *et al.*, 2002; Yoshimatsu *et al.*, 2000). It has been suggested that these bacteria are already adapted to the host intestinal environment, thus being able to colonize other sites directly that they could not colonize otherwise (Wiles *et al.*, 2005). In the present work, although bacteria were not directly transmitted among mice, they still showed a differential colonization pattern. This probably indicates an adaptation to the murine gut environment. Besides, a more generalized and persistent intestinal colonization could imply a greater disruption of the epithelial barrier, thus facilitating the passage of Stx to systemic circulation. The molecular basis that explains this differential pattern of colonization should be studied further.

The analysis of the *Xba*I restriction patterns proved the clonal relationship between the stool-recovered strains and the parent strain. Also, the serotype and the genotype were verified and their identity confirmed. However, stool-recovered strains showed a decrease in Stx2 production compared to the parent strain. Stx2 is encoded in a lambdaoid prophage, under the control of phage promoters that modulate the lytic cycle (Mühldorfer *et al.*, 1996). Therefore, the production of Stx is significantly enhanced when the phage is induced by several stress signals, such as antibiotic exposure (Grif *et al.*, 1998), norepinephrine (Lyte *et al.*, 1996), and hydrogen peroxide or neutrophils as a consequence of activating the bacterial SOS response to DNA damage (Wagner *et al.*, 2001a). In addition, Stx2 expression is under the control of an antiterminator, which acts as a transcriptional activator, and it has been demonstrated that its over expression results in increased Stx production (Neely & Friedman, 1998). Several reports have examined the relationship between antiterminator expression and Stx production. In fact, it has been

demonstrated that mutations in this antiterminator region are responsible for decreased or even null Stx expression (Koitabashi *et al.*, 2006; Wagner *et al.*, 2001b; Zhang *et al.*, 2010). Besides, it has been postulated that the SOS response could regulate and co-ordinate the expression of Stx and type III secretion (T3S) systems (Garmendia *et al.*, 2005) to potentiate colonization (Tree *et al.*, 2009). Therefore, one of these regulatory mechanisms could have been affected during the passage through the gastrointestinal tract of mice in our model, thus explaining the increased pathogenicity observed in stool-recovered strains in spite of the lower Stx2 production. In addition, the mentioned decrease could explain the fact that stool-recovered strains administered at optimal doses did not cause an increased mortality in mice. On the other hand, it has been demonstrated that the Stx cytotoxicity is increased by LPS *in vivo* (Keepers *et al.*, 2006; Palermo *et al.*, 2000; Sauter *et al.*, 2008). In addition, an increased resistance to unfavourable host intestinal environment has been related to an upregulation of genes involved in LPS biosynthesis and its transport to the cell surface (Snyder *et al.*, 2004). However, it is unlikely that a mechanism involved in LPS synthesis may account for the differences observed among bacterial strains in our model because stool-recovered strains did not show an increased LPS content per cell compared to the parent strain (data not shown). Further research will be necessary to completely elucidate the underlying mechanism (genetic or epigenetic) involved in the pathogenesis and the bacterial adaptation to the murine intestinal environment.

Finally, our results show that stool-recovered strains are able to persist, multiply and colonize the intestinal microenvironment when significantly lower inocula were used. Moreover, the use of a 10<sup>4</sup>-fold-reduced inoculum was still able to induce death in a percentage of mice, in spite of the lower Stx2 production. These results suggest that bacteria adapted to the host intestinal environment may have a lower infective dose, thus facilitating intestinal colonization and environmental dissemination. Taken together, our findings in the HUS mouse model provide evidence for the adaptation of a human STEC strain to the mouse intestine, thus representing a useful tool for the investigation of STEC pathogenesis.

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