FACTORS AFFECTING THE LETHALITY OF CAMPYLOBACTER FETUS SUBSPECIES JEUNI IN MICE

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SUMMARY. Intraperitoneal injection of Campylobacter fetus ss. jejuni into HAM/ICR mice was lethal, but viable counts of bacteria from whole body homogenates, organs and blood indicated that death was not due to sustained bacterial multiplication. Heat-killed organisms (5 × 10⁹ cfu) injected into 7-day-old mice caused death within 24 h and this was shown to be due to endotoxin. Both ferric iron and heterologous lipopolysaccharide enhanced virulence; the LD₅₀ was lowered from 1.8 × 10⁹ cfu to 2.7 × 10⁷ cfu when both were used. Three-day-old or adult animals survived challenge with Campylobacter fetus without clinical symptoms when challenged orally or by intravenous or intraperitoneal routes.

INTRODUCTION

In 1946, Levy isolated from the diarrhoeal stools of patients suffering from gastro-enteritis, vibrio organisms which were similar to Vibrio jejuni from bovine diarrhoeal infections (Jones, Orcutt and Little, 1931). Vibrio jejuni was subsequently called Campylobacter fetus ss. jejuni (Smibert, 1978) and was implicated as the cause of a haemorrhagic diarrhoea in children (Middelkamp and Wolf, 1961; Wheeler and Borchers, 1961) and of acute enteritis in adults (Mandel and Ellison, 1963). Both C. fetus ss. jejuni and ss. intestinalis are now recognised as causing respiratory, vascular, nervous, gastro-intestinal and reproductive tract infections in man (Philip and Tilton, 1977). They cause haemorrhagic congestion and mucoid oedema of the intestinal mucosa (Lambert et al., 1979). Systemic infections in man are usually characterised by bacteraemia (Jackson, Hinton and Allison, 1960; Hallett, Botha and Logan, 1977; Guerrant et al., 1978; Robinson, 1978).

Procedures for isolating C. fetus ss. jejuni have been improved (Butzler et al., 1973; Skirrow, 1977) but it has been difficult to find a suitable animal model to study the pathogenesis of campylobacter enteritis (Prescott and Karmali, 1978; Butzler and Skirrow, 1979; Ng, 1981). Diarrhoea was induced in 3-day-old chickens after oral administration of C. fetus ss. jejuni 9 × 10⁷ cfu/ml (Ruiz-Palacios, Escamilla and Torres, 1981) although other investigators have failed to confirm this (Manninen, Prescott and Dohoo, 1982).

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In the present investigation, the initial aim was to develop a mouse model to study campylobacter infection, but our results indicate that the lethality of \textit{C. fetus ss. jejuni} for young mice is not directly correlated with the organism's replication in this host. Hence, the mouse is an unsuitable model of infection in man.

**Materials and Methods**

\textbf{Campylobacter strains.} \textit{C. fetus ss. jejuni} strain 5636 isolated from the stools of a child with diarrhoea was provided by Dr M. B. Skirrow, Worcester Royal Infirmary, Worcester. \textit{C. fetus ss. jejuni} strain 4249 isolated from the stools of an adult with diarrhoea was provided by Dr T. F. Elias-Jones of the former City Laboratory, Glasgow.

\textbf{Growth and harvesting of organisms.} Bacteria were grown in Nutrient Broth No. 2 (Oxoid) 25g/L supplemented with yeast extract (Difco) 5g/L and L-cystine (Sigma Chemical Co., St Louis, MO) 0.2g/L. This medium (100 ml) was seeded with 10-0 ml of a 24-h starter culture grown in the same medium. Flasks were incubated in anaerobic jars in an atmosphere of O$_2$ 5%, CO$_2$ 10%, and N$_2$ 85% (v/v) at 37°C on an orbital shaker set at 100 rpm (L.H. Engineering, Stoke Poges, Bucks).

The organisms were removed from the medium by centrifugation at 12,000 g for 15 min at 4°C. The cell pellets were resuspended in 0.85% (w/v) sterile physiological saline to provide the bacterial suspensions used in animal inoculations. The number of viable organisms in the inoculum was determined (Miles, Misra and Irwin, 1938) with 6.0% horse-blood-agar plates (Columbia agar base 3.9 g/100 ml plus defibrinated horse blood 6 ml; Oxoid) incubated at 37°C in the atmosphere described above.

\textbf{Heat treatment of \textit{C. fetus ss. jejuni} cells.} Samples of the bacterial suspension were heated at 100°C for 30 min, centrifuged at 12,000 g for 15 min at 4°C and the supernate removed aseptically. The killed organisms were resuspended in sterile physiological saline. The efficiency of heat treatment was checked by plating out the supernates and resuspended pellets and demonstrating sterility.

\textbf{Sonication of \textit{C. fetus ss. jejuni} cells.} In order to obtain preparations containing cytoplasmic constituents or cell envelopes, a thick suspension of \textit{C. fetus} organisms was treated in an M.S.E. ultrasonic machine for 5 min in an ice-bath. The sonicated cells were centrifuged at 12,000 g for 15 min and the supernate and pellet were retained.

\textbf{Mice.} HAM/ICR mice (1-35-day-old) were obtained from the outbred colony in the Microbiology Animal Unit. Siblings were used from pens containing a single breeding pair. The animals were provided with a standard pelleted diet and water \textit{ad libitum}.

\textbf{Administration of \textit{C. fetus ss. jejuni} to mice.} With 3-5-week-old mice, 0.5 ml of the bacterial suspension was administered orally directly into the stomach through a nylon intravenous cannula (pink luer 3FG or green luer 2FG; Portex Ltd, Hythe, Kent). Intravenous (i.v.) injections (0.2 ml) were given into the tail vein. For intraperitoneal (i.p.) challenge, a volume of 0-5 ml was injected.

In delivering i.p. challenges, we took account of the small size of very young mice. A constant number of organisms was given to 1-3-day-old mice in 0.05 ml and to 7- and 14-day-old mice in 0.1 ml of the suspending fluid. This was achieved by standardisation against the 5th International Opacity Reference Preparation designated as 10 International opacity units (o.u) and obtained from the World Health Organization, Laboratory for Biological Standards (N.I.B. S.C., Holly Hill, Hampstead, London). In our experience, 1 o.u. was equivalent to $1 \times 10^9$ cfu of \textit{C. fetus ss. jejuni}/ml.

\textbf{Determination of numbers of viable \textit{C. fetus ss. jejuni} organisms surviving in 7-day-old mice.} Mice were killed by cervical dislocation at 12-h intervals, washed in ethanol and blotted dry with paper tissue. With a mixer emulsifier (Silverson Machines Ltd, London), the whole body was minced and homogenised in 10-0 ml of sterile saline to give a fine suspension. The homogenates were centrifuged for 10 min at 1500 g to remove large particles, and the number of viable \textit{C. fetus ss. jejuni} cells in the supernate was determined; the values were expressed as cfu/mouse of 2.5 g average weight.

The selective medium for counting \textit{C. fetus ss. jejuni} in the mouse homogenates was Columbia agar base plus 6% lysed blood, vancomycin HCl 10 µg/ml (Eli Lilly and Co. Ltd, ...
Basingstoke, Hants), polymyxin B sulphate 2·5 IU/ml and trimethoprim lactate 5 µg/ml (Wellcome Reagents, Beckenham, Kent).

The effect of ferric ammonium citrate on C. fetus ss. jejuni in 7-day-old mice. A sterile solution of ferric ammonium citrate 2·0 mg/ml (=Fe³⁺ 320 µg/ml; BDH Chemicals Ltd, Poole, Dorset) in saline was prepared and different concentrations in 0·1 ml were injected i.p. with the viable suspension of C. fetus ss. jejuni into 7-day-old mice. The mortalities were recorded during a 10-day period. The survival of the organisms was determined in a separate experiment by counting viable cells in whole-body homogenates, as described above.

RESULTS

Effect of age on susceptibility of HAM/1CR mice to C. fetus ss. jejuni

Initially, 3- and 5-week-old mice were given 3 × 10⁹ cfu of C. fetus ss. jejuni either orally or by i.v. injection, but none of these mice developed diarrhoea and none died. When younger mice were challenged i.p. with 3 × 10⁹ cfu of C. fetus ss. jejuni strain 5636, three of 20 1-day-old and six of 20 7-day-old mice died within 10 days. This effect was particularly evident after the C. fetus strain was passaged five times in 5-week-old mice; the organisms were isolated from the spleen at each passage before re-inoculation into uninfected mice.

In an attempt to define the influence of age on susceptibility, mice of various ages (1–35 days) were challenged i.p. with (1·25–10·0) × 10⁹ cfu of C. fetus ss. jejuni strain 5636. During a 10-day post-injection period, it was apparent that 7-day-old mice were most susceptible; 32 of 40 died and the LD₅₀ was 1·8 × 10⁹ cfu (table I). By contrast, the LD₅₀ values for younger and older mice were significantly higher.

Recovery of viable C. fetus ss. jejuni from whole body homogenates

After i.p. injection of (0·62–2·5) × 10⁹ cfu into groups of 7-day-old mice, pairs of animals were killed at 12-h intervals and the numbers of viable campylobacters in whole-body homogenates were determined. After the injection of sub-lethal numbers

<table>
<thead>
<tr>
<th>Age of mice*</th>
<th>LD₅₀ (and 95% confidence limits) expressed in cfu (10⁹)</th>
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<tbody>
<tr>
<td>(days)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8·0 (5·0, 12·0)</td>
</tr>
<tr>
<td>3</td>
<td>4·4 (3·3, 5·8)</td>
</tr>
<tr>
<td>7</td>
<td>1·8 (1·3, 2·4)</td>
</tr>
<tr>
<td>14</td>
<td>2·0 (1·5, 2·7)</td>
</tr>
<tr>
<td>21</td>
<td>15·0 (10·0, 23·0)</td>
</tr>
<tr>
<td>35</td>
<td>&gt; 15·0 ...</td>
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* Each group contained 10 mice. The statistical analyses were done as described by Boyd (1956). In comparing LD₅₀ values from two groups of mice, the difference is judged significant at the P = 5% level if the LD₅₀ of one group lies outside the 95% confidence limits of the other.
of *C. fetus ss. jejuni*, the organisms were eliminated as shown in fig. 1. When the higher dose of $2.5 \times 10^9$ cfu was injected, $<10\%$ of the bacteria were recovered in the whole-body homogenates after 48 h.

**Susceptibility of 7-day-old mice to live and heat-killed *C. fetus ss. jejuni* strain 5636**

The i.p. injection of $1 \times 10^{10}$ cfu of *C. fetus ss. jejuni* into 7-day-old mice killed all the animals within 48 h. When the same number of organisms was killed by heating at $56^\circ$C for 30 min before injection, 14 out of 15 of the challenged mice died. When the number of living or dead organisms in the injection dose was varied from $0.62 \times 10^9$ to $10 \times 10^9$ cfu, mortality rates were similar at the highest dose, 100% and 93.3%
Fig. 2.—Probit mortality rates plotted against the number of living or dead C. fetus ss. jejuni cells in the i.p. challenge dose. The heavy arrows represent doses that gave 0% or 100% mortality and therefore probit values cannot be plotted. The LD50 value for living cells was $1.48 \times 10^9$ cfu and for dead cells was $3.8 \times 10^9$ organisms. Probit values were calculated according to Boyd (1956).

respectively. With $2.5 \times 10^9$ cfu, 86% of the animals were killed with live cells and 73.3% with dead cells. A plot of the probit of mortality against injection dose of living or dead C. fetus ss. jejuni showed parallel dose-response curves which suggests a similar mode of action (fig. 2), i.e., that both live and dead bacteria caused toxic death, with the live challenge reaching the lethal level after limited multiplication in vivo.

A suspension of $1 \times 10^{10}$ dead cells of C. fetus ss. jejuni strain 5636 in 0.1 ml killed nine of 10 7-day-old mice after i.p. injection. On the other hand, the supernate from $1 \times 10^{10}$ cells (0.1 ml), obtained after centrifugation of killed cells at 12,000 g for 15 min, failed to kill any of the challenged animals. Moreover, i.p. challenge with 0.1 ml of the supernate from a sonicated suspension of $1 \times 10^{10}$ cells of C. fetus ss. jejuni centrifuged at 12,000 g for 15 min did not cause the death of 7-day-old mice, whereas i.p. injection of 0.1 ml containing the pellet of cell debris from $1 \times 10^{10}$ organisms killed 65% of the animals.

**Enhancement of lethal toxicity of C. fetus ss. jejuni by ferric ammonium citrate**

Intraperitoneal injection of 4–20 µg of iron in the form of ferric ammonium citrate into 7-day-old mice caused no apparent ill-effects, whereas 100 µg killed 60% of the
32

D. E. S. STEWART-TULL, F. K. P. NG AND A. C. WARDLAW

FIG. 3.—The effect of ferric iron on the lethality of *C. fetus* ss. *jejuni* strains 4929 and 5636. The LD50 for strain 4929 was lowered from $3\times10^9$ to $8.5\times10^8$ cfu with 12 µg ferric iron; with strain 5636 the same amount of iron lowered the LD50 value from $1.78\times10^9$ to $4.5\times10^8$ cfu in 7-day-old mice. The LD50 values were calculated by the procedure of Boyd (1956).

animals. The incorporation of increasing amounts of ferric ammonium citrate ($Fe^{3+}4-20 \mu g$) into the challenge dose of $1\times10^9$ cfu of *C. fetus* ss. *jejuni* strain 5636 or strain 4249 significantly decreased the LD50 values (fig. 3). The LD50 value of the *C. fetus* ss. *jejuni* strain 5636 suspension was $1.78\times10^9$ cfu, but this was lowered to $4.5\times10^8$ cfu in the presence of 12-0 µg of ferric iron. With *C. fetus* ss. *jejuni* strain 4249, the LD50 value for the suspension alone was $3.0\times10^9$ cfu and for the suspension plus 12 µg of ferric iron it was $8.5\times10^8$ cfu.

The addition of ferric iron 40-120 µg/ml to 100 ml of nutrient broth containing yeast extract and cysteine did not significantly increase the growth of *C. fetus* ss. *jejuni*. In experiments in which viable organisms were recovered from whole-body homogenates, the counts were consistently higher in animals given 12-0 µg of ferric iron and
LETHALITY OF C. FETUS SS. JEJUNI FOR MICE

33

C. fetus ss. jejuni i.p.; in some experiments, a two-fold log_{10} difference was noted. On the other hand, there was a progressive decrease in the viable counts in homogenates from 7-day-old mice challenged i.p. with the C. fetus ss. jejuni suspension alone. The elimination of bacteria from mice given 8–12 μg of ferric iron was delayed for at least 24 h; then there was a reduction in the number of organisms recovered.

Similar elimination patterns in two groups of animals after i.p. challenge with \(6 \times 10^8\) cfu were obtained until 18 h, when one group was given an injection of \(Fe^{3+}\) 12 μg which retarded the elimination of C. fetus ss. jejuni (fig. 4). In addition, the elimination of organisms was less pronounced in animals given 12 μg of ferric iron at time 0 and a further 12 μg at 18 h. The incorporation of 12 μg of ferric iron into the inoculum did not increase the toxicity of heat-killed C. fetus ss. jejuni for the 7-day-old mice.

Fig. 4.—The effect of ferric iron on the reisolation of viable C. fetus ss. jejuni after i.p. injection into 7-day-old mice. Each value represents the mean viable count from two mice killed at each time. ○—○ \(6 \times 10^8\) cfu injected; ■—■ \(6 \times 10^8\) cfu injected and \(Fe^{3+}\) 12 μg injected 18 h later; □—□ \(6 \times 10^8\) cfu and \(Fe^{3+}\) 12 μg injected at time 0; ■—■ \(6 \times 10^8\) cfu and \(Fe^{3+}\) 12 μg injected together at time 0 and a further \(Fe^{3+}\) 12 μg injected 18 h later.
When 7-day-old mice were challenged i.p. with 25–400 μg of Shigella flexneri lipopolysaccharide (LPS) the LD50 was 61.5 μg (95% C.L. 39.7, 95.4) and in the presence of 12.0 μg of ferric iron the LD50 was 46.6 μg (95% C.L. 33.2, 65.5). The injection of 25 μg of LPS did not cause the death of any animals, although sometimes there was some shivering and ruffling of the coats for 1–2 h. Consequently, to avoid these effects, LPS was administered 4 h before the challenge with (0.078–1.3) x 10⁹ cfu of C. fetus ss. jejuni strain 5636. There was a 22-fold reduction in the LD50 value in the presence of 25 μg of LPS. However, in animals given 25 μg of LPS before challenge with C. fetus ss. jejuni and 12 μg of ferric iron, there was a 66-fold reduction in the LD50 value when compared with the bacterial challenge alone (table II).

**TABLE II**
The effect of ferric ammonium citrate and heterologous S. flexneri LPS on the lethal toxicity of C. fetus ss. jejuni for 7-day-old mice

<table>
<thead>
<tr>
<th>Nature of challenge*</th>
<th>LD50 (and 95% confidence limits) expressed in cfu (10⁹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial challenge alone</td>
<td>180 (70, 450)</td>
</tr>
<tr>
<td>Fe³⁺12 μg with challenge</td>
<td>45 (20, 99)</td>
</tr>
<tr>
<td>LPS 25 μg 4h before challenge</td>
<td>8.1 (3.4, 19)</td>
</tr>
<tr>
<td>LPS 25 μg 4h before, plus Fe³⁺12 μg with challenge</td>
<td>2.7 (0.9, 7.7)</td>
</tr>
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</table>

* All groups of 20 mice were challenged i.p. with a dose in the range (0.08–1.3) x 10⁹ cfu of C. fetus ss. jejuni.

**DISCUSSION**

In an attempt to establish a model of campylobacter infection in the HAM/1CR mouse, initial experiments were done with intraperitoneal challenges in animals of different ages. Mice in the age range 7–14 days were significantly more susceptible than either 1–3-day-old animals or young adults (> 21-day-old). However, none of the animals examined post mortem showed signs of diarrhoea, and faecal pellets could be seen in the rectum, indicating that there had been no gross disturbance of normal bowel function. In a separate study viable organisms were recovered from the blood, liver and spleen up to 7 days after i.v., i.p. or oral administration to 3–5-week-old mice. Although there was evidence of a transitory period of bacterial multiplication, C. fetus ss. jejuni strains 4249 and 5636 were not recovered from the blood or spleen after 48 h. The largest numbers of organisms were recovered from the liver, but by the seventh day after infection viable C. fetus ss. jejuni was not isolated (Ng, 1981). The rapid elimination of C. fetus ss. jejuni from the blood indicated that death of the 7–14-day-old mice was a consequence of the total number of bacteria in the initial inoculum and not of rapid in-vivo multiplication.

After the injection of smaller numbers of C. fetus ss. jejuni, recovery experiments from whole-body homogenates showed that the number of viable organisms decreased...
up to 48 h. With larger challenge doses there was a slower rate of elimination which indicated some in-vivo multiplication, but this was insufficient to overcome the normal clearance mechanism. Although 90% of the C. fetus ss. jejuni challenge was eliminated after 48 h (fig. 1), the mice still died.

The resistance of 1- and 3-day-old mice may be associated with antibacterial factors such as lactoferrin or lysozyme from maternal milk. The phagocytic activity of reticuloendothelial cells in mice was shown to be well developed by 21 days (Benacerraf et al., 1954). Immunocompetence is related to the formation of antigen-recognising macrophages (Argyris, 1968) and this might explain the resistance of the older mice. Such cells are not well developed in young mice, and this may be an additional factor in the susceptibility of 7- and 14-day-old animals. It is interesting that a similar pattern of age susceptibility was observed with staphylococcal infection in mice (McKay and Arbuthnott, 1979).

The factor responsible for the death of the 7-day-old mice challenged with C. fetus ss. jejuni seemed to be endotoxin, as animals also died after the injection of similar numbers of heat-killed cells. Toxicity was related to the total number of heat-killed cells injected and this suggested that a critical quantity of endotoxin caused death of the young mice. Sufficient endotoxin was present in $5 \times 10^9$ organisms to cause death of 7-day-old mice within 24 h. Although 7-day-old mice were shown to destroy C. fetus ss. jejuni organisms after sub-lethal challenges with fewer organisms, it seemed that there was some bacterial multiplication. However, when the critical amount of endotoxin had accumulated in the animal, death occurred.

The importance of iron in microbial infections is now well recognised (Bullen, Rogers and Griffiths, 1978; Bullen, 1981), as also is the fact that iron in host tissues may not be freely available to bacteria in an infection (Morgan, 1974). For instance, in cultures containing serum, there may be a bactericidal or bacteriostatic effect for some organisms. These effects can be reversed by saturating the iron-binding capacity of serum transferrin with $Fe^{3+}$; in vivo the administration of enough ferric ammonium citrate to saturate the transferrin increased the lethality of Escherichia coli (Bullen and Rogers, 1969). The injection of 4-12 $\mu$g ferric iron produced a similar effect in 7-day-old mice challenged with strains of C. fetus ss. jejuni by i.p. injection.

Although many studies have examined the effect of $Fe^{3+}$ ions, it is possible that $NH_4^+$ ions might have an effect, so it would be interesting to examine the effects of a series of ferric and ammonium compounds on C. fetus ss. jejuni. The citrate is unlikely to be important because considerable levels are normally found in blood (Wolcott and Boyer, 1948).

The precise mechanism whereby the ferric iron and endotoxin prevents elimination of campylobacter organisms is complex and uncertain. Saturation of iron-binding proteins, e.g., transferrin and lactoferrin, could leave some free $Fe^{3+}$ and thus allow growth of the bacteria. Macrophages will readily remove ferric-lactoferrin complexes from the circulation (Van Snick, Masson and Heremans, 1974). The results presented here also suggest that the ferric iron did not impair phagocytic cell function because elimination of organisms still occurred (fig. 4). It was concluded that the recovery curves represented the balance between limited bacterial multiplication and phagocytosis.

Hypoferraemia may occur during infection and may enhance non-specific resistance to bacteria (Cartwright et al., 1946). The addition of iron as ferric
ammonium citrate may reverse this situation. In addition, the injection of 10 µg of *E. coli* LPS caused hypoferremia in adult mice after 12 h and then the serum iron appeared to increase progressively (Baker and Wilson, 1965). Kampschmidt and Upchurch (1962) found that 100 µg of LPS caused a maximum decrease in serum iron 8–16 h after injection in rats. Endotoxin may induce a non-specific resistance to infection with some bacteria. In the present study the injection of *S. flexneri* LPS 4 h before challenge with *C. fetus ss. jejuni* significantly increased the mortalities. Some decrease in serum iron could be expected after 4 h, but the decrease would be most marked after 24 h with a return to normal values after 48 h (Kampschmidt and Upchurch, 1964). The observed increase in susceptibility of the young 7-day-old mice to *C. fetus ss. jejuni* in the presence of LPS might be due to an endotoxin-induced neutropaenia. Sub-lethal doses of 0·62 × 10⁹ cfu of *C. fetus ss. jejuni* 5636 caused the death of all animals in the presence of the heterologous LPS 2·5 mg/kg live weight. The LD₅₀ was reduced from 1·8 × 10⁹ cfu without LPS to 8·1 × 10⁷ cfu with LPS. The combined effects of *S. flexneri* LPS and ferric ammonium citrate reduced this even more to 2·7 × 10⁷ cfu. It seems that competition between the mouse and *C. fetus ss. jejuni* for iron is only one factor in the endotoxin-induced death. The influence of the *C. fetus ss. jejuni* LPS on the balance between free and bound iron remains to be examined.

In adult immunocompetent mice, it seems likely that phagocytic clearance overcame the campylobacter challenge and a lethal dose of endotoxin did not accumulate. On the other hand, in 7-day-old mice given sub-lethal doses of *C. fetus ss. jejuni* together with ferric iron, endotoxin accumulated more rapidly and death occurred before the phagocytic cells could remove the organism.

Recently, Blaser *et al.* (1983) reported that adult HA-1CR mice given 1 × 10⁸ cfu of *C. fetus* orally were bacteraemic after 10 min. These results were presented in a way that makes it difficult to assess whether bacterial multiplication occurred or not. These authors agreed that no experimentally infected adult mice showed overt illness or died. In the present study, the examination of whole body homogenates from young mice indicated that there is a reduction in numbers of viable organisms with time and elimination of organisms; this agrees with the findings of Field, Underwood and Berry (1982). However, it does seem that the HAM/1CR mouse is an unsuitable model for studies of campylobacter infection.

### REFERENCES


LETHALITY OF C. FETUS SS. JEJUNI FOR MICE


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