Sex Differences in Susceptibility of ICR Mice to Oral Infection with *Corynebacterium kutscheri*

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Abstract: Sex difference in susceptibility to oral infection with *Corynebacterium* (*C.*) *kutscheri* was experimentally studied in ICR mice. Immature (4-week-old) and adult (14-week-old) mice were inoculated with two infecting doses of *C. kutscheri*, and necropsied for bacteriological and serological survey 4 weeks after the bacterial infection. No macroscopic lesions at necropsy were demonstrated, except for one adult male given 10^9 bacteria. In immature mice, *C. kutscheri* isolated from the oral cavity and cecum with FNC agar, were recovered in only 40.0% of female mice but in 90.0% of male mice given 10^6 bacteria ($p < 0.05$), and in only 55.6% of female mice but in 80.0% male mice given 10^8 bacteria. In adult mice given 10^9 bacteria, the organism were recovered in only 45.5% of female mice but in 90.9% of male mice ($p < 0.05$), furthermore, the mean number of organisms in the cecum of male mice harboring the organism was significantly higher than that in females ($p < 0.01$). Castration caused an increase in host resistance in adult male mice. These results indicated that ICR male mice were more susceptible than females, in terms of bacterial colonization in the cecum and the oral cavity, to oral infection with *C. kutscheri*.

Key words: *C. kutscheri*, ICR mice, sex difference

Introduction

Numerous mammalian species, including human beings, show sex differences in susceptibility to infectious diseases. Several observations suggest superior capability of the female in resistance to some bacteria and in immunization with various antigens. In *Corynebacterium* (*C.*) *kutscheri* infection, intravenous infection failed to detect the sex difference in terms of mortality in Swiss Lynch and C57BL/6 mice [16]. But we ob-

served that the organism most frequently colonized the alimentary canal, mainly the oral cavity, cecum, and colon and rectum in subclinically infected mice and rats, and indicated that the organism is transmitted mainly via the fecal-oral route [2, 4]. In our previous study, differences in susceptibility of mice among various strains to oral infection with *C. kutscheri* [3] were revealed by using a powerful tool established in our laboratory for the isolation of *C. kutscheri* from subclinically infected mice and rats [2, 4, 5]. In the present

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study, we set out to investigate the sex difference in susceptibility of ICR mice to oral infection with *C. kutscheri* bacteriologically and serologically.

Materials and Methods

Animals: Male and female Slc:ICR SPF mice, which were resistant to oral infection with *C. kutscheri* [3], were purchased from Japan SLC, Inc. (Shizuoka), at 3 and 10 weeks of age for the immature and adult group, respectively. After one or four weeks quarantine and acclimation under study conditions, the animals were assigned to this study. All the mice were housed in polycarbonate cages (W22 × D33 × H13 cm) in groups of 5 animals in each cage. They were kept in an animal room under controlled conditions of 20–25°C temperature, 40–70% relative humidity, and a 14 hr (5:00–19:00) lighting cycle. Commercial diet (MB-1, Funabashi Farm Co. Ltd., Chiba, Japan) and water were provided *ad libitum*.

Inoculation with *C. kutscheri*: The strain S5L [5] of *C. kutscheri* isolated from the lung abscess of a rat was used. Frozen stock culture of the strain was thawed and cultured on heart infusion agar (HIA: Eiken Chemical Co., Ltd, Tokyo, Japan) for 48 hr at 37°C. Serial 10-fold dilutions of the bacterial suspension were then prepared with heart infusion broth (HIB: Eiken Chemical Co., Ltd., Tokyo, Japan). All the mice were inoculated orally with 0.2 ml of each dilution of the suspension adjusted to a concentration of 1.7×10^6 or 7.0×10^8 CFU/0.2 ml for the immature group, and 1.2×10^6 or 3.0×10^9 CFU/0.2 ml for the adult group. All the mice were observed for clinical signs and were humanely killed with ether, bled by cardiac puncture and necropsied at 4 weeks after inoculation. The animals used in this study were treated and killed in accordance with the provisions for animal welfare of The Nippon Veterinary and Animal Science University which followed the Guidelines for Animal Experimentation issued by the Japanese Association for Laboratory Animal Science [12].

Bacteriology: A selective medium, furazolidone nalidixic acid- colimycin agar (FNC agar) [5], was used for the isolation of *C. kutscheri*. The mucous membrane of the oral cavity, nasal cavity and trachea was wiped with a fine cotton swab moistened with sterile HIB and streaked onto FNC agar. The cecum was

homogenized in 10 volumes of sterile HIB with a glass mortar. Serial 10-fold dilutions of the homogenates were prepared in HIB, and a 0.05 ml aliquot of each dilution was placed on FNC agar. After incubation of the agar plates at 37°C for 72 hr, *C. kutscheri* colonies were enumerated. The average of counts was used to calculate the number of bacteria (CFU/g). Isolates were identified with Gram's stain and an agglutination test with rabbit antiserum prepared against strain S5L [5].

Agglutination test for antibody to *C. kutscheri*: A blood sample was collected from the heart of each mouse at necropsy. Serum was diluted 1:5 with phosphate-buffered saline (pH 7.2). Agglutination tests for serum antibody to *C. kutscheri* were performed by using a microtiter system as described previously [21] and a commercial antigen (Denka Co., Tokyo, Japan). Sera lacking agglutination at a 1:5 dilution were designated as antibody negative.

Castration: Both testes of 11-week-old male mice were excised after incision along the median line of the lower abdomen. For sham-gonadectomized male mice, a midline incision was made in the lower abdomen and the testes were pulled out and then reinstated. For sham-gonadectomized female mice, a midline incision was made in the back skin and the ovaries were pulled out and then reinstated. The skin incision was closed with sutures. Three weeks after the surgery, all of the mice were experimentally infected with *C. kutscheri* as stated above. This test were carried out twice.

Statistical analysis: Statistical evaluation of the prevalence of *C. kutscheri* in females and males was conducted by means of Fisher's exact test. To compare the mean numbers of the organisms in the cecum, Student's *t*-test was used.

Results

The presence of clinical signs and macroscopic lesions, and the change in body weight: No clinical signs were observed in any of the mice during the observation period. At necropsy, one male mouse in the adult group given 10^9 bacteria had an abscess in the kidney which was yellowish-white and 0.7 mm in diameter. In the other mice, no macroscopic lesions were found at necropsy. The body weight of none of the mice decrease noticeably during the observation period.

Isolation of *C. kutscheri* and serum antibody: Isola-

Table 1. Bacteriological and serological survey in ICR mice inoculated with *C. kutscheri* at 4 weeks post inoculation

Age of mice	Inoculum dose (CFU)	Sex	Recovery of <i>C. kutscheri</i>	Serum antibody ^{a)}
Immature	1.7×10^6	Female	4/10 ^{b)} (40.0)	1/10 (10.0) 1:10
		Male	9/10 (90.0)	0/10 (0)
	7.0×10^8	Female	5/9 (55.6)	0/9 (0)
		Male	8/10 (80.0)	2/10 (20.0) 1:10, 1:20
Adult	1.2×10^6	Female	0/10 (0)	0/10 (0)
		Male	0/10 (0)	0/10 (0)
	3.0×10^9	Female	5/11 (45.5)	0/11 (0)
		Male	10/11 (90.9)	3/11 (27.3) 1:5–1:40

a) By agglutination test, antibody titer. b) Number positive/Number tested (%). c) Statistically significant difference at $*p < 0.05$ (Fisher's exact test).

Fig. 1. The number of *C. kutscheri* per gram of cecum at 4 weeks post inoculation. Each value represents the mean $\pm$ S.D. in mice. a) Number of mice. b) Statistically significant difference at $**p < 0.01$ (Student's *t*-test). c) Not detected.

tion of *C. kutscheri* and serum antibody to *C. kutscheri* determined by agglutination test are shown in Table 1. In the immature groups, *C. kutscheri* were recovered in 4 of 10 female mice (40.0%) and in 9 of 10 male mice (90.0%) in a group given 10^6 bacteria, in 5 of 9 female mice (55.6%) and in 8 of 10 male mice (80.0%) in a group given 10^8 bacteria. In these male groups, the prevalence of *C. kutscheri* would appear to be higher than in the female groups, and values in the male group given 10^6 bacteria was significantly higher than that in the female groups ($p < 0.05$). In adult groups, no organisms were recovered in either female or male mice groups given 10^6 bacteria, but in groups given 10^9 bacteria, *C. kutscheri* were recovered in 5 of 11 female mice (45.5%) and 10 of 11 male mice (90.9%). The

prevalence of *C. kutscheri* in the male group was significantly higher than that in female group ($p < 0.05$). The organisms were recovered from the cecum in most of the mice harboring *C. kutscheri*. The isolation rate was next highest in the oral cavity, followed by the trachea, but was low in the nasal cavity. The mean number of *C. kutscheri* per gram of cecum are shown in Fig. 1. In immature groups, the value for male mice inclined to be higher than that for female mice, but there were no significant differences between female and male mice given 10^6 or 10^8 bacteria. On the other hand, in adult mice given 10^9 bacteria, the mean number of the organisms in male mice ($10^{3.9}$ CFU/g) was significantly higher than that in female mice ($10^{2.1}$ CFU/g) ($p < 0.01$).

Table 2. Bacteriological and serological survey in castrated adult ICR mice inoculated with *C. kutscheri* at 4 weeks post inoculation

Inoculum dose (CFU)	Operation	Recovery of <i>C. kutscheri</i>	Serum antibody ^{a)}	
0.64–1.1 × 10 ⁹	Castration	3/9 ^{b)} (33.3)	1/9 (11.1)	1:10
	Sham male	10/10 (100.0)	3/10 (30.0)	1:160–320
	Sham female	5/10 (50.0)	2/10 (20.0)	1:5, 1:10

a) By agglutination test, antibody titer. b) Number positive/Number tested (%). c) Statistically significant difference at ** p<0.01 (Fisher's exact test).

Fig. 2. The number of *C. kutscheri* per gram of cecum in castrated adult ICR mice at 4 weeks post inoculation. Each value represents the mean ± S.D. in mice. a) Number of mice. b) Statistically significant difference at *p<0.05, **p<0.01 (Student's *t*-test).

Seroconversion to *C. kutscheri* by agglutination test was detected in 1 of 10 female mice (10%, titer: 1:10) given 10⁶ bacteria and in 2 of 10 male mice (20%, titer: 1:10 or 1:20) given 10⁸ bacteria in the immature groups, and in 3 of 11 male mice (27.3%, titer: 1:5–1:40) given 10⁹ bacteria in the adult group. That was not detected in adult mice given 10⁶ bacteria. There were no significant differences between female and male mice in the serological response.

The effect of castration on host resistance (Table 2, Fig. 2): The body weights of the all animals did not show an extreme decrease after operation, and no macroscopic lesions were demonstrated at necropsy. When *C. kutscheri* was inoculated at a dose of 0.64–1.1 × 10⁹ CFU, castration of the adult male group significantly reduced the prevalence of *C. kutscheri* and the number of organisms recovered from the cecum at 4 weeks

post inoculation, compared with the sham-operated male group (p<0.01). The number of *C. kutscheri* in castrated males was at nearly the same level as that in sham-operated female mice. And the prevalence and the number of *C. kutscheri* in the sham-operated male group were significantly higher than those in the sham-operated female group (p<0.05, p<0.01). In seroconversion to *C. kutscheri* in the agglutination test, there were no significant differences between three groups, but titers in the sham-operated male group inclined to be higher than in the castration and sham-operated female groups.

Discussion

This is the first report demonstrating sex difference in susceptibility of ICR mice to oral infection with *C. kutscheri*. Immature and adult ICR male mice showed higher susceptibility than female ICR mice as defined by the number of mice harboring *C. kutscheri*. And in adult the mice, the mean number of the organisms in the cecum in male mice was significantly greater than that in female mice. Furthermore, castration increased the resistance of adult ICR male mice to *C. kutscheri* infection to almost the same level as that of female ICR mice and the reappearance of those sex differences was observed in the adult mice. These results indicated that male ICR mice were more susceptible to oral infection with *C. kutscheri* than females in terms of bacterial colonization in the cecum and oral cavity. In future it will be necessary to examine these sex differences in another mouse strain.

To our knowledge, the demonstration of sex differences in the susceptibility of mice to bacterial infection have been reported in mycobacterial infection [22, 23], and males were more susceptible to this infections. The challenge route in these models was intravenous or in-

traperitoneal. On the other hand, a sex difference was demonstrated in the route of oral infection in our study. It is interesting that the sex difference in bacterial infection would be considered to be associated with indigenous bacterial flora and/or oral immunity. Pierce-Chase *et al.* [16] observed that the survival of Swiss Lynch and C57BL/6 mice in intravenous infection was independent of the age or sex of the animals. This inconsistency may be attributable to differences mainly in the challenge route and experimental conditions, such as the mouse strain and inoculation size. But in the case of natural infection with *C. kutscheri* in wild voles (*Microtus agrestis*), it was suggested that the prevalence of infection with gross lesions in the lungs was significantly greater in males than in females although the numbers sampled were not large [7]. As the present authors suggested that *C. kutscheri* is transmitted mainly via the fecal-oral route in mouse [4], there could be a sex difference in the susceptibility of mice to natural infection.

The prevalence of *C. kutscheri* in immature groups of given 10^6 bacteria was higher than that in adult groups. It is interesting to note the age difference in the susceptibility of ICR mice to oral infection with *C. kutscheri*, but as for the details, we shall wait to see what the future holds.

Sex differences in host resistance of mice to infection with some of bacteria, viruses and parasites are recognized, that is males are often more susceptible to infection with *Mycobacterium (M.) intracellulare* [23], *M. marinum* [22], vesicular stomatitis virus [6], coxsackie virus [11], hepatitis B virus [1], *Leishmania (L.) major* [13], *L. mexicana* [18], *Brugia pahangi* [15], *Plasmodium chabaudi* [8], *Giardia muris* [9] and *Paracoccidioides brasiliensis* [19]. On the other hand, females are more susceptible to infection with *Schistosoma mansoni* [10] and *Toxoplasma gondi* [17]. In some of these models, castration of highly susceptible male mice increased their resistance, and testosterone treatment at a physiological dose in the castrated mice suppressed resistance [8, 13, 14, 22]. Furthermore, a large number of studies have observed the multiple effects of estrogens on infectious processes [20]. Further studies concerning the relationship between *C. kutscheri* infection and sex hormones are needed to clarify the sex differences in the mice.

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