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Cadmium Chloride Susceptibility, a Characteristic of *Campylobacter* spp.

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We report a simple diagnostic characteristic useful in the presumptive identification of *Campylobacter jejuni* and *Campylobacter coli*. Filter paper disks impregnated with cadmium chloride were placed on streaked agar medium. Zones of growth inhibition for *Campylobacter* spp. occurred at 1.25 μg per disk. Other enteropathogens (*Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Escherichia coli*, and *Yersinia enterocolitica*) were resistant to at least 40 μg per disk, with the exception of a strain of *Shigella flexneri*, which showed first susceptibility at 10 μg per disk. Most of the 52 *Campylobacter* strains, which were isolated from human clinical and animal sources, showed zones of inhibition greater than 10 mm with 2.5 μg of cadmium chloride per disk. At 20 μg per disk, *Campylobacter* isolates from clinical sources were significantly ($P < 0.01$) more susceptible to cadmium chloride inhibition than were those from meat samples.

The mechanism by which *Campylobacter jejuni* (11) expresses virulence in human gastroenteritis is obscure partly because reliable experimental models for studying the human infection are limited. A mouse model for campylobacteriosis has been difficult to develop because adult mice exhibit a high degree of resistance to enteritis with this bacterium. We have reported on a weanling mouse model for studying the pathogenesis of the organism (9), but the dose required to induce diarrhea in mice is greater than human infectious doses.

Several approaches have been applied successfully to enhance the apparent virulence of infectious agents. These include manipulation of immune status by treating animals with environmental toxicants and immunosuppressive agents (10, 13–15), including chronic exposure of mice to cadmium (2, 4, 7). Effects of cadmium chloride exposure on the immune system in humans have also been reported (6, 12).

During attempts to enhance susceptibility of weanling BALB/c mice to food and clinical isolates of *C. jejuni* by intoxication with cadmium chloride, we discovered a diagnostic characteristic useful in the presumptive identification of *Campylobacter* spp.

MATERIALS AND METHODS

Preliminary animal handling. Weanling (3- to 6-week-old) BALB/c mice of both sexes were maintained on antibiotic-free food (Charles River Mouse Chow; Agway, Syracuse, N.Y.) and tap water. Before intragastric or intraperitoneal inoculation, fecal specimens from representative mice in each group were suspended in sterile distilled water and cultured on two consecutive days to ensure that the mice were not carriers of *C. jejuni* or related species of *Campylobacter*.

Bacterial cultures. *Campylobacter* strains (Table 1) were identified by standard criteria (8) and were cultured on brucella broth (Difco Laboratories, Detroit, Mich.) with 2% agar (Bacto-Agar; Difco) and 5% laked horse blood (Oxoid, Ltd., London) with an antibiotic supplement (3) for 24 h at 37 or 42°C in a 5% O_2–10% CO_2–85% N_2 environment. All strains had been passed at least twice on laboratory media. Cultures were maintained frozen (−70°C) in brucella broth containing 20% glycerol for use as stocks and in subsequent comparisons. Other enteric bacteria (Table 1) were grown aerobically at 37°C on brucella agar plates.

Cadmium immunosuppression. The mouse 50% lethal dose of cadmium chloride (Fisher Scientific Co., Fairlawn, N.J.) was estimated by intraperitoneal injection of groups of mice (eight per group) with twofold dilutions (400 to 12.5 μg in 0.5 ml per mouse). The 50% lethal dose was determined to be 75 μg per mouse. To establish immunosuppression, weanling BALB/c mice were given three intraperitoneal injections (of cadium chloride (10 μg) per week for 3 weeks. Preliminary tests showed that such treatments induce a 90% reduction in hemolytic PFU per spleen after immunization with sheep erythrocytes as a test antigen, as compared with immunized, untreated mice (8,900 versus 92,000 PFU per spleen; unpublished data).

Animal challenge. Bacterial suspensions for challenges were made in brucella broth by the removal of growth from 24- to 48-h brucella agar plates with a sterile swab. After appropriate dilutions, *C. jejuni* (10^7 CFU per mouse) was injected in cadmium-treated and untreated mouse groups by both intragastric and intraperitoneal routes. We confirmed the number of bacteria injected by plating serial 10-fold dilutions of the inoculum on brucella agar. The animals were observed for 3 weeks for signs of disease (9) or for death. Attempts were made to isolate bacteria from feces, peritoneal fluid, liver, and spleen.

In vitro cadmium chloride susceptibility assays. From a stock solution of cadmium chloride (1 mg/ml in sterile distilled water) 20-μl dilutions containing 20, 10, 5, 2.5, and 1.25 μg of cadmium chloride were prepared and applied to sterile 6-mm filter paper disks (Schleicher & Schuell, Inc., Keene, N.H.). The disks were dried at room temperature and stored at 4°C until use. One loopful of approximately 10^5 to 10^6 cells was streaked onto the surfaces of brucella agar plates, and the disks were aseptically transferred and pressed into the agar surface. The plates were incubated at 42°C under a microaerobic atmosphere for 48 h, and zones of inhibition were measured.

MICs of cadmium chloride preventing growth of *C. jejuni* strains were estimated in agar medium by incorporating

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concentrations ranging from 1.25 to 100 μg/ml and inoculating the agar with 10^5 to 10^6 CFU of *C. jejuni* strains. The MIC was defined as the lowest concentration of cadmium chloride causing suppression of visible bacterial growth after an overnight incubation at 42°C.

RESULTS

After 3 weeks of exposure to cadmium, treated mice challenged with *C. jejuni* did not exhibit any clinical signs, weight loss, or spontaneous death. After intragastric and intraperitoneal injections with *C. jejuni*, no significant differences in susceptibility to *C. jejuni* were seen between controls and treated mice. No *C. jejuni* could be isolated from cadmium-treated mice after 24 h. Therefore, we attempted to determine whether tissue levels of cadmium chloride were preventing growth of the injected *C. jejuni*.

Campylobacter strains were found to be highly susceptible to very low levels of cadmium chloride. Most of these strains gave a zone of inhibition of more than 10 mm with a 2.5-μg disk (Fig. 1), coinciding with the MIC of 2.5 μg/ml found for several strains of *C. jejuni* tested in agar medium with incorporated cadmium. A total of 48 strains of *C. jejuni*, *C. coli*, *C. laridis*, four other species of *Campylobacter*, and 18 enteric bacteria commonly found in clinical stool samples were screened for cadmium susceptibility with impregnated disks (Fig. 2). Differences in susceptibility were observed between isolates from clinical sources (n = 10) and from meat sources (n = 38) (Table 2). The average zones of inhibition were, respectively, 38.4 and 28.1 mm when the isolates were subjected to 20 μg of cadmium chloride per disk. Although the standard deviations overlap, the differences in the clustering of these data were significant at $P \leq 0.01$ (5). At 2.5 μg per disk, the average zones of inhibition were 20.8 and 17.2 mm, respectively, and the differences were significant at the 5% level. Non-*C. jejuni* and non-*C. coli* species of *Campylobacter* were also highly susceptible to 2.5 μg of cadmium chloride, inlcuding *C. laridis* (*C. jejuni* G-6) (1), which was resistant to tetracycline and nalidixic acid. In contrast, all other enteropathogens tested were resistant to at least 20 μg per disk, with the exception of one strain of *S. flexneri* which showed first susceptibility at 10 μg per disk. Most of the enteric bacteria tolerated 100 μg of cadmium chloride per disk.

TABLE 1. Bacterial cultures, original animal sources, and suppliers

Organism	Source (no.)	Donor[a] (no.)
Campylobacter jejuni	Human (9)	a (2), b (1), c (2), d (1), e (1), f (1), g (1)
Campylobacter laridis	Human (1)	d (1)
Campylobacter jejuni	Chicken (15)	h (15)
Campylobacter jejuni	Lamb (12)	h (12)
Campylobacter coli	Pork (10)	f (1), h (10)
Campylobacter fetus	Human (1)	b (1)
Campylobacter fetus subsp. *fetus*	Sheep (1)	b (1)
Campylobacter fetus subsp. *intestinalis*	Human (1)	b (1)
Campylobacter fetus subsp. *venerealis*	Cow (1)	b (1)
Escherichia coli	Human (1)	i (1)
Salmonella typhi	Human (3)	i (1), j (2)
Salmonella typhimurium	Human (2)	i (1), j (1)
Shigella flexneri	Human (2)	i (1), j (1)
Shigella sonnei	Human (3)	k (3)
Vibrio cholerae	Human (1)	l (1)
Vibrio cholerae, non-O1	Human (1)	l (1)
Vibrio parahaemolyticus	Human (2)	l (2)
Yersinia enterocolitica	Human (2)	j (2)

[a] a, Naval Medical Research Institute, Bethesda, Md.; b, American Type Culture Collection, Rockville, Md.; c, R. Kazmi, Children's Hospital, Washington, D.C.; d, C. Bachurski, Georgetown University Hospital, Washington, D.C.; e, R. Hawley, University of Maryland, College Park, Md.; f, D. Rozak-McDonnell, University of Maryland, College Park, Md.; g, M. Doyle, Food Research Institute, Madison, Wis.; h, Meat Science Research Laboratory, Beltsville, Md.; i, J. Johnson, Prince Georges General Hospital, Cheverly, Md.; j, T. Cook, University of Maryland, College Park, Md.; k, A. Huq, International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh; l, M. Voll, University of Maryland, College Park, Md.

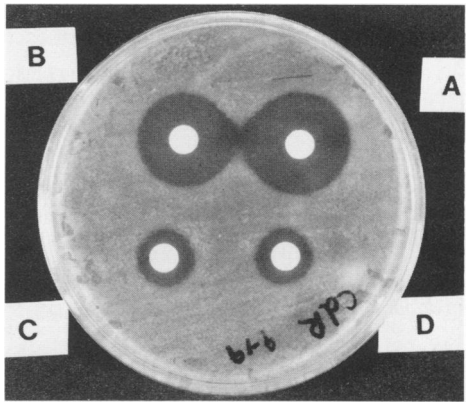

FIG. 1. Growth inhibition of *C. jejuni* by cadmium chloride. Each disk was impregnated with 20 (A), 10 (B), 5 (C), or 2.5 (D) μg of cadmium chloride and applied to streaked agar plates.

DISCUSSION

The use of immunosuppressive levels of cadmium chloride did not enhance susceptibility to infection with *C. jejuni* by either intraperitoneal or intragastric challenge. The extremely high susceptibility of *C. jejuni* to very low levels of cadmium chloride undoubtedly contributed to the complete

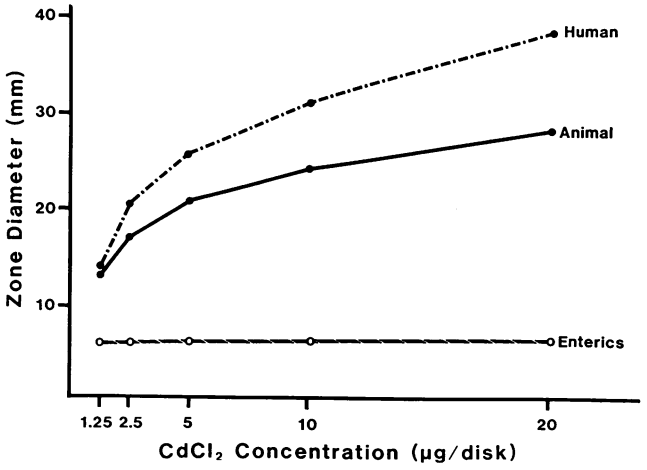

FIG. 2. Susceptibility of *C. jejuni*, *C. coli*, and enteric bacteria to cadmium chloride. Campylobacter isolates tested were 10 clinical and 38 meat isolates. Enteric bacteria were 18 representative strains of *Shigella* spp., *Salmonella* spp., *Yersinia* spp., *Vibrio* spp., and *E. coli*. The minimum zone size (6 mm) is the disk diameter.

TABLE 2. Zones of inhibition surrounding a disk containing cadmium chloride for the indicated groupings of bacteria

Organism (no.)	Source	*n*	Zone diam (mm; mean ± SD) for amt of $CdCl_2$/disk (μg):	
			2.5	20
Campylobacter jejuni (9)/*Campylobacter laridis* (1)	Human	10	20.8 ± 4.9[a]	38.4 ± 7.9[a]
Campylobacter jejuni (27)/*Campylobacter coli* (11)	Animals	38	17.2 ± 4.4[b]	28.1 ± 5.4[b]
Non-*Campylobacter jejuni*/non-*Campylobacter coli Campylobacter* sp.	Varied	4	16.8 ± 0.5[b]	25.5 ± 2.5[b]
Salmonella spp., *E. coli*, *Shigella* spp., *Vibrio* spp., *Y. enterocolitica*	Human	18	6.0 ± 0[c]	6.4 ± 0.9[c]

[a,b,c] Data with different superscript letters in the same column are significantly different at $P < 0.05$ for the disks with 2.5 μg of $CdCl_2$ and at $P < 0.01$ for the disks with 20 μg of $CdCl_2$ as determined by the Duncan multiple range statistic (5). The minimum zone (6 mm) is the disk diameter.

inhibition of infection with *C. jejuni* in our mice. Although we failed to isolate a cadmium-resistant strain of *C. jejuni* (to test the general usefulness of immunosuppression for inducing *Campylobacter* enteritis in mice), our results strongly suggest that cadmium susceptibility could be used differentially for diagnostic identification of this bacterium in stools and food isolates.

Isolates of *C. jejuni* from clinical sources were more susceptible to cadmium chloride than those from meat samples. This observation suggests a provocative hypothesis: either the strains in human infection do not have zoonotic origins or a transformation in cadmium susceptibility occurs during pathogenesis. Although differences in cadmium susceptibility were observed, serological and epidemiological evidence implicates foods of animal origin in human campylobacteriosis. Because of this evidence, we suggest the latter hypothesis as more likely. In our mouse model, virulence is enhanced through animal passage (9), and it is probable that virulence-mediating components are induced during pathogenesis. The enhanced cadmium susceptibility of the clinical isolates may be a direct or indirect function of the induction of this component. Such a virulence-enhancing component may be associated with increased uptake or toxicity of cadmium chloride for the organism. Demonstration of the process could be useful in describing specific virulence determinants elaborated by *C. jejuni* and *C. coli*.

Because most of the enteric bacteria that cause diarrhea or dysenterylike infections (*Shigella* spp., *Salmonella* spp., *Yersinia* spp., *Vibrio* spp., and *Escherichia coli*) are highly resistant to cadmium chloride, the presence of *C. jejuni* in stool or food samples can be attested to by the inhibition of growth around a 2.5-μg cadmium chloride disk. Nalidixic acid disk susceptibility is widely used by diagnostic laboratories to aid in identifying *C. jejuni* and *C. coli* but is not a reliable method for *C. laridis* diagnosis because the organism is resistant to nalidixic acid (1). Cadmium chloride susceptibility is a stable feature which may prove useful as a diagnostic or differential characteristic between *Campylobacter* spp. and other potential enteropathogens. If environmental and tissue levels of cadmium are sufficient, this metal also could play a role in the epidemiology and ecology of *C. jejuni* infections.

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