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The Chicken Embryo as a Model for Campylobacter Invasion: Comparative Virulence of Human Isolates of *Campylobacter jejuni* and *Campylobacter coli*

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Eleven-day-old chicken embryos were used to compare the relative virulence of minimally passaged human isolates of *Campylobacter jejuni* and *Campylobacter coli*. Graded doses of bacteria were inoculated onto the chorioallantoic membrane, and 50% lethal doses were calculated at 72 h postinfection. Strains varied markedly in their ability to invade the chorioallantoic membrane and kill the embryos. The 50% lethal doses varied by about 6 logs for 25 strains of *C. jejuni*, and by 2 logs for 5 strains of *C. coli*. Although both outbred and inbred embryos were employed in the study, the latter were found to be more susceptible to infection with most strains. All isolates were screened for plasmid DNA, but there was no apparent relationship between plasmid content and virulence of strains for the embryos. Neither could virulence be associated with the production of siderophores by the strains. The ability of selected strains of *C. jejuni* to invade the liver of embryos was also studied. The number of campylobacters culturable from the liver was found to be inversely related to the 50% lethal dose of the strain. By inoculating 11-day-old embryos intravenously, it was possible to demonstrate that a strain of *C. jejuni* which was poorly virulent after chorioallantoic inoculation was relatively noninvasive. Invasiveness alone, however, could not fully account for the lethality of two highly virulent strains of *C. jejuni* administered by the intravenous route. Finally, there was no correlation between motility and virulence in this model system.

Campylobacter jejuni and *Campylobacter coli* are now known to be major world-wide causes of bacterial enterocolitis in humans (4, 5, 29). Despite the fact that these organisms cause a variety of diarrheal diseases, the exact mechanisms involved in pathogenesis have not been defined. There is both clinical and experimental evidence to suggest that invasion or production of enterotoxins or both play a role in the disease process (5, 17, 22, 26, 27, 29, 31, 34, 41). The contribution, however, of other potential virulence factors, including adhesins (9, 34), cytotoxins (22, 27, 36, 53), outer membrane proteins (47), and siderophores is yet to be established.

C. jejuni is clearly an invasive organism. Overt colitis is a common clinical presentation, and many patients have blood, mucus, and leukocytes in their stools. There is also evidence of local invasion in experimentally infected animals. *C. jejuni* has been shown to invade the intestinal epithelial cells of orally inoculated infant chickens (5, 40, 45, 51), infant mice (37), and hamsters (21). Systemic invasion into the blood and other extraintestinal organs has been documented in both humans (5, 28, 29, 48) and experimental animals (3, 7, 15, 45, 49, 51). Although strains have been found to be uniformly negative when tested by the Sereny test (30, 34), invasion has been demonstrated with in vitro tissue culture systems (5, 36, 37).

Davison and Solomon (10) were the first to use the chicken embryo as an animal model to study the invasive capacity of *C. jejuni* strains. They inoculated 10^6 organisms onto the chorioallantoic membrane (CAM) of 11- to 17-day-old Ross Tint embryos and measured the recovery of bacteria from the heart and liver 24 and 48 h postinoculation. Campylobacters were recovered from the organs of embryos over the whole age range tested, but there was a progressive decrease in the numbers recovered with increasing embryonic age. The two strains investigated displayed different abilities to invade the CAM, and differences were most pronounced in 11-day-old embryos. These workers concluded that *C. jejuni* possesses the ability to invade the epithelium of the highly vascularized CAM, thereby entering the embryonic circulation. They suggested that by inoculating 11-day-old chicken embryos and examining them 48 h later it should be possible to compare the pathogenicity of different strains of *C. jejuni*.

The studies reported here confirm and extend the observations made by Davison and Solomon (10). Eleven-day-old chicken embryos inoculated chorioallantoically were used to compare the virulence of human isolates of *C. jejuni* and *C. coli*. The results indicate that such isolates vary markedly in their ability to invade the CAM and kill the embryos. Additional data presented herein, however, suggest that differences in invasive capacity cannot entirely account for the differences in virulence displayed by the strains. These findings establish the value of the chicken embryo as a potential model for the analysis of virulence factors in campylobacter strains.

MATERIALS AND METHODS

Bacterial strains. The *C. jejuni* and *C. coli* strains used in this study were kindly provided by the following individuals: George Buck, Department of Pathology, University of Texas Medical Branch, Galveston; G. Ann Hébert, Hospital Infections Program, Centers for Disease Control, Atlanta, Ga.;

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George Morris, Enteric Bacteriology Section, Centers for Disease Control; Diane Newell, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, United Kingdom; Gloria Pierce, Clinical Enterics Section, Texas Department of Health Laboratory, Austin; Paul Southern, University of Texas Health Science Center, Dallas; and Joy G. Wells, Enteric Bacteriology Section, Centers for Disease Control. Upon receipt in our laboratory, strains were passaged only once or twice on brucella agar (BA) containing 4% defibrinated sheep blood before being frozen in brucella broth (BB) with 15% glycerol for use as stock cultures.

Strains were confirmed to be *C. jejuni* in our laboratory if they were oxidase and catalase positive, showed darting motility, were sensitive to nalidixic acid and resistant to cephalothin, grew at 37 and 42°C but not at 25°C, and hydrolyzed hippurate using the procedure of Hébert et al. (20). *C. jejuni* INN-1 and INN-73, which were received from G. Morris, were originally obtained from G. M. Ruiz-Palacios, Institute Nacional de la Nutricion, Tlalpan, Mexico. The enterotoxic properties of these two strains have been well documented (26, 41). The *C. coli* strains used in this study were identified to the species level at the Centers for Disease Control by the technique of DNA hybridization. None was able to hydrolyze hippurate when tested in our laboratory. Strain 517, which was identified as *C. jejuni* in previous publications (12, 13), was reidentified as a strain of *C. coli* at the Centers for Disease Control by DNA hybridization.

Culture medium and growth conditions. BB was prepared by using the following formula (ingredients per liter of distilled water): tryptone, 10 g; peptamin, 10 g; dextrose, 1 g; yeast extract, 2 g; sodium chloride, 5 g. BA consisted of BB which was solidified with 1.5% purified agar. All components except sodium chloride were purchased from Difco Laboratories (Detroit, Mich.). Plates were routinely incubated at 37°C in a microaerobic growth environment as previously described (13).

Broth cultures of *C. jejuni* and *C. coli* were grown by using a biphasic culture system similar to that described by Rollins et al. (39). BA (20 ml) was dispensed into a 500-ml Erlenmeyer flask and allowed to solidify. This was overlaid with 20 ml of BB containing approximately 10^7 to 10^8 CFU of *C. jejuni* or *C. coli* per ml. Inocula for the liquid phase of the biphasic cultures were obtained by harvesting the growth after 24 h from a BA plate containing 4% defibrinated sheep blood. After inoculation, flasks were capped with Bioshield sterile wrap (American Scientific Products, McGaw Park, Ill.) and placed inside Ziploc bags (Dow Chemical Co., Indianapolis, Ind.). The bags were evacuated (three times) and refilled each time with a mixture of 90% nitrogen and 10% carbon dioxide. Flasks were incubated at 37°C without shaking.

Selection of motile and nonmotile variants. *C. jejuni* strain 1376 was grown in biphasic liquid culture, and appropriate dilutions were plated in BA containing 0.5% agar. After incubation at 37°C for 72 h, both motile and nonmotile colony types were identified in the soft agar plates. Nonmotile organisms formed small opaque colonies with compact edges. Motile organisms formed either large diffuse colonies (highly motile cells) or large opaque colonies which were surrounded by diffuse haloes (motile cells). The last colony type was shown to consist of only motile cells rather than a mixture of motile and nonmotile cells by plating individual clones in soft agar. Both a highly motile and a nonmotile variant were selected by picking individual colonies and inoculating them into the center of soft agar plates. Nonmotile variants were selected by picking cells from the centers of small opaque colonies, whereas motile variants were selected by picking cells from the outside edges of large diffuse colonies. Each motility variant was passaged 15 times in soft agar plates; after the last passage, colonies were streaked onto BA with 4% sheep blood, incubated for 24 h, and frozen in BB–15% glycerol for use in experiments.

The phenotype of each variant was confirmed by direct observation of hanging drop preparations and by transmission electron microscopy of negatively stained cells. The motile variant was flagellated, whereas the nonmotile variant was aflagellated.

Chicken embryo methodology. (i) Chicken embryos. Both outbred and inbred chicken embryos were used in these studies. Fertile eggs from an outbred flock of White Leghorn hens (Ideal no. 236) were obtained from Ideal Poultry Co., Cameron, Tex. Embryonated eggs from an inbred line of chickens (Hy-Line Variety SC) were purchased from Hy-Line International, Dallas Center, Iowa. Eggs were incubated for 11 days at 37°C in a rotary egg incubator (model 5; Petersime Incubator Co., Gettysburg, Ohio).

(ii) Chorioallantoic inoculation of embryos. Strains of *C. jejuni* or *C. coli* were grown in biphasic culture medium at 37°C to the middle to late log phase (optical density at 625 nm, 0.5 to 1.0) and harvested by centrifugation at 7,000 × *g* for 10 min at 4°C. Cell pellets were gently suspended in BB to obtain an initial density of 1×10^9 to 3.0×10^9 CFU/ml. Serial decimal dilutions were prepared in BB, and embryos were inoculated with 0.1 ml of each dilution on the CAM after the creation of an artificial air space. At least five embryos were used per dose, and experiments were repeated at least once. Control embryos inoculated with BB were included in each experiment.

After inoculation, embryos were incubated in a horizontal position at 37°C and were candled daily for 3 days to determine viability. Embryos found dead after infection were cultured to establish that no organisms other than *C. jejuni* or *C. coli* were present. Mean lethal dose (LD_{50}) values were calculated by the method of Reed and Muench (38).

(iii) Intravenous inoculation of embryos. Intravenous inoculations were carried out by a previously described technique (14). Briefly, a rectangular window (approximately 2 by 5 mm) was cut in the shell over a prominent vein by using a hand drill fitted with an abrasive disk. The shell flap was then lifted off with an 18-gauge needle. All windows were removed 1 h before inoculation. In the interim, eggs were held at 37°C. Inoculations were performed while candling by using a tuberculin syringe fitted with a 27-gauge needle. Graded doses of *C. jejuni* were administered in a volume of 0.1 ml, and control embryos received 0.1 ml of BB. After infection, eggs were incubated at 37°C, and viability was determined as described above.

(iv) Assessment of systemic invasion. Eleven-day-old Hy-Line Variety SC chicken embryos were inoculated chorioallantoically with either 2×10^7 or 2×10^6 campylobacters, and systemic invasion into the liver was determined after 24 h. Livers from infected embryos were removed aseptically, placed in 1 ml of BB, weighed, and homogenized. The number of CFU per 100 mg of tissue was determined by plating serial 10-fold dilutions onto a selective medium (18). The medium used consisted of BA plus 4% defibrinated sheep blood containing the following antibiotics: amphotericin B (1 mg/liter; Sigma Chemical Co., St. Louis, Mo.), cefoperazone (7.5 mg/liter; Pfizer, Inc., New York), colistin

(5,000 IU/liter; Sigma), and rifampin (5 mg/liter; Sigma). Plates were incubated at 37°C and were counted after 48 h.

Plasmid analysis. Strains of *C. jejuni* and *C. coli* were grown overnight at 37°C on BA–4% blood, and plasmid DNA was extracted by the procedure of Birnboim and Doly (2). Lysates were examined by electrophoresis in 0.9% agarose gels.

Siderophore synthesis. A bioassay was used to screen *C. jejuni* and *C. coli* strains for the ability to produce siderophores in low-iron medium. The details of this assay are described in an accompanying paper (11).

Statistics. Statistical significance was determined by the Student *t* test.

RESULTS

Virulence of *C. jejuni* and *C. coli* strains for White Leghorn chicken embryos. Eleven-day-old White Leghorn chicken embryos were used to compare the virulence of minimally passaged human isolates of *C. jejuni* and *C. coli*. Graded doses of bacteria ranging from 10^8 to 10^1 CFU per embryo were inoculated onto the CAM, and LD_{50}s were calculated on the basis of deaths that occurred through 72 h postinfection. All values represent the pooled results of at least two replicate experiments, each with five embryos inoculated per dose of bacteria. The LD_{50}s for 25 strains of *C. jejuni* and 5 strains of *C. coli* are shown in Table 1. Strains varied markedly in their ability to invade the CAM and kill the embryos after 3 days of incubation. There was at least a 6-log difference between the least virulent (LD_{50}, $>3.3 \times 10^8$) and most virulent (LD_{50}, 2.2×10^2) *C. jejuni* strain tested. Although the range was not as great with the comparatively small sample of *C. coli* strains, LD_{50}s varied from 6.4×10^6 to 1.9×10^4, more than a 2-log difference.

TABLE 1. Virulence of *C. jejuni* and *C. coli* strains for 11-day-old chicken embryos inoculated chorioallantoically

Campylobacter strain	LD_{50} at 72 h		Plasmid carriage	Siderophore production
	White Leghorn	Hy-Line Variety SC		
C. jejuni				
6324	$>3.3 \times 10^8$	$>1.7 \times 10^8$	−	−
1213	$>2.8 \times 10^8$	3.9×10^7	−	−
A2999	$>2.2 \times 10^8$	$>1.7 \times 10^8$	−	−
7831	$>2.0 \times 10^8$	NT[a]	−	+
LOPEZ	$>1.0 \times 10^8$	$>1.5 \times 10^8$	+	+
295470	1.6×10^8	NT	−	+
296597	8.9×10^7	NT	+	−
281346	5.8×10^7	NT	−	+
3503	4.0×10^7	8.8×10^7	+	+
1250	3.2×10^7	NT	−	+
81116	2.1×10^7	NT	−	−
UT-1	2.1×10^7	4.4×10^6	−	−
1270	1.5×10^7	2.5×10^6	−	+
INN-73	6.1×10^6	NT	−	−
INN-1	5.6×10^6	NT	−	−
850	3.9×10^6	NT	−	−
ADC-1	2.0×10^6	NT	−	−
1376	1.3×10^6	8.2×10^3	−	+
3629	4.4×10^5	NT	+	−
BE5-1636	4.0×10^5	NT	−	−
BE5-1373	1.3×10^5	NT	−	−
BE5-246	8.5×10^4	1.5×10^5	−	+
19437	2.0×10^4	2.0×10^3	−	−
BE5-1638	5.0×10^3	NT	−	−
289504	8.0×10^2	3.1×10^2	−	−
303955	NT	1.4×10^2	−	−
C. coli				
F1753	6.4×10^6	NT	−	+
F1789	2.6×10^5	NT	+	−
2718	7.4×10^4	NT	−	+
517	6.1×10^4	2.7×10^3	−	+
B2015	1.9×10^4	6.2×10^3	+	−

[a] NT, Not tested.

TABLE 2. Daily mortality of 11-day-old Hy-Line Variety SC chicken embryos after chorioallantoic inoculation with different doses of three strains of *C. jejuni*

Strain and dose (CFU)	Deaths/total at indicated time:			LD_{50} at 72 h
	24 h	48 h	72 h	
A2999				$>1.7 \times 10^8$
1.7×10^8	0/10	3/10	4/10	
1.7×10^7	0/9	0/9	0/9	
1.7×10^6	0/9	0/9	0/9	
1.7×10^5	0/5	0/5	0/5	
1.7×10^4	0/5	0/5	0/5	
BE5-246				1.5×10^5
3.4×10^7	0/5	2/5	5/5	
3.4×10^6	0/10	1/10	10/10	
3.4×10^5	0/10	0/10	7/10	
3.4×10^4	0/10	0/10	1/10	
3.4×10^3	0/10	0/10	0/10	
3.4×10^2	0/10	0/10	0/10	
3.4×10^1	0/5	0/5	0/5	
289504				5.0×10^2
2.0×10^6	0/5	4/5	5/5	
2.0×10^5	0/10	8/10	10/10	
2.0×10^4	0/10	4/10	10/10	
2.0×10^3	0/10	3/10	10/10	
2.0×10^2	0/10	0/10	3/10	
2.0×10^1	0/10	0/10	2/10	

Each strain of *C. jejuni* and *C. coli* was examined for the presence of plasmid DNA to determine whether the invasive phenotype correlated with plasmid carriage. Only five of the *C. jejuni* strains and two of the *C. coli* strains carried plasmids (Table 1), and there was no apparent relationship between plasmid content and virulence of strains for chicken embryos.

Since the production of siderophores is thought to play an important part in the virulence of certain invasive pathogens (16), each of the *C. jejuni* and *C. coli* strains was screened for the ability to produce siderophores in vitro. There was no obvious correlation between virulence for chicken embryos and production of siderophores by the strains (Table 1).

Virulence of selected *C. jejuni* and *C. coli* strains for Hy-Line Variety SC chicken embryos. Outbred White Leghorn embryos were found to be useful for determining the relative virulence of most *C. jejuni* and *C. coli* strains. With three of the strains, however, uneven dose responses were obtained, which complicated the calculation of LD_{50}s. Such variability might simply be due to genetic differences inherent in embryos from an outbred line of chickens. Alternatively, since most commercially raised poultry have campylobacters in their intestinal flora (4), some embryos could possess maternal antibodies which are capable of reacting with antigenic determinants on the infecting organisms. Thus, the virulence of selected *C. jejuni* and *C. coli* strains was tested in embryos from an inbred line of chick-

TABLE 3. Invasion of the liver by *C. jejuni* strains 24 h after chorioallantoic inoculation of 11-day-old Hy-Line Variety SC chicken embryos

Strain	Inoculum size (CFU)	Mean CFU/100 mg (wet weight) of liver ± SEM (no. of embryos inoculated)
LOPEZ	2.1×10^7	$3.8 \times 10^2 \pm 1.5 \times 10^2$ (8)
A2999	2.2×10^7	$2.3 \times 10^3 \pm 1.2 \times 10^3$ (10)
1376	2.9×10^7	$9.7 \times 10^4 \pm 2.1 \times 10^4$ (10)
303955	2.1×10^7	$1.4 \times 10^5 \pm 0.75 \times 10^5$ (8)
289504	1.9×10^7	$4.7 \times 10^6 \pm 2.6 \times 10^6$ (10)
LOPEZ	2.1×10^6	$1.2 \times 10^2 \pm 1.1 \times 10^2$ (8)
A2999	2.2×10^6	$3.6 \times 10^2 \pm 1.1 \times 10^2$ (10)
1376	2.9×10^6	$6.4 \times 10^3 \pm 1.7 \times 10^3$ (10)
303955	2.1×10^6	$1.5 \times 10^5 \pm 0.66 \times 10^5$ (8)
289504	1.9×10^6	$3.2 \times 10^5 \pm 1.1 \times 10^5$ (10)

ens. The Hy-Line Variety SC bird is an F_1 cross of two moderately inbred White Leghorn lines. Both parental populations are fixed for the B2 haplotype of the Ea-B blood group. They are vaccinated against a variety of viral pathogens and are maintained free of *Mycoplasma* species. Their exposure to *C. jejuni* or *C. coli* is not known.

The results obtained when 12 *C. jejuni* strains and 2 *C. coli* strains were tested in these embryos is shown in Table 1. With *C. jejuni* 6324, A2999, and LOPEZ, the type of embryo did not influence the LD_{50}. All of these strains were poorly virulent, with LD_{50}s in excess of 10^8. The LD_{50} for strain BE5-246 was also approximately the same for both types of embryos. With nine other strains tested, however, Hy-Line Variety SC embryos were more susceptible to infection by anywhere from 2.5- to 150-fold. *C. jejuni* 3503 was the only strain tested in which the LD_{50} for Hy-Line Variety SC embryos was greater than that for White Leghorn embryos. In this case, the difference between them was only about twofold. In all subsequent experiments, Hy-Line Variety SC embryos were employed because of their greater susceptibility to infection and their genetic homogeneity.

Time and dose-response results with three strains of *C. jejuni*. Typical time and dose-response results with strains of low, intermediate, and high virulence are presented in Table 2. The timing of the deaths with each of the three strains indicates that the infection is progressive. Although no embryo died at 24 h postinoculation, at 48 h the number of deaths was directly related to the virulence of the strain administered. Since the majority of embryos infected with virulent strains were dead within 72 h, this time was chosen for calculating the LD_{50}s.

It should be noted from the mortality data in Table 2 that in no case did more deaths occur in any group of embryos given a dose smaller than the preceding one. It is our experience that with outbred animals, including White Leghorn embryos, such inconsistencies in dose responses are sometimes seen. This emphasizes the value of using inbred chicken embryos for studies of this type.

Invasion of the liver of 11-day-old Hy-Line Variety SC chicken embryos by selected strains of *C. jejuni*. Experiments were performed to determine whether there was a correlation between the LD_{50}s of different strains of *C. jejuni* and their ability to invade the liver of chicken embryos (Table 3). Five strains of *C. jejuni* which varied widely in their LD_{50}s were chosen for these studies. Strains LOPEZ and A2999 were selected because of their low virulence (LD_{50}s in excess of 10^8), whereas strains 1376, 289504, and 303955 were chosen because of their comparatively high virulence (LD_{50}s between 8.2×10^3 and 1.4×10^2). Groups of embryos were inoculated chorioallantoically with either 2×10^7 or 2×10^6 organisms; 24 h later livers were excised, and the mean CFU per 100 mg (wet weight) of tissue was determined. The number of *C. jejuni* organisms culturable from the liver was inversely related to the LD_{50} of the strain administered (Table 3). This relationship was true with both sizes of inocula used. With the larger inoculum, all values were statistically significant at $P < 0.001$, except when strains 1376 and 303955 were compared. With the smaller inoculum, all values were statistically significant at $P < 0.001$, except for the values of strains 303955 and 289504 (these were significant at $P < 0.01$).

Figure 1 contains data that expand on those given in Table 3. Embryos were infected on the CAM with 2×10^7 CFU of strain LOPEZ, 1376, or 289504. The number of *C. jejuni* organisms in the embryonic liver was followed at 6-h intervals from the time of inoculation until the experiment ended at 24 h. The nearly exponential increase in CFU seen with strains 289504 and 1376 contrasted sharply with the delayed increase seen with strain LOPEZ. At each time point there was a statistically significant difference in values between strains ($P < 0.001$).

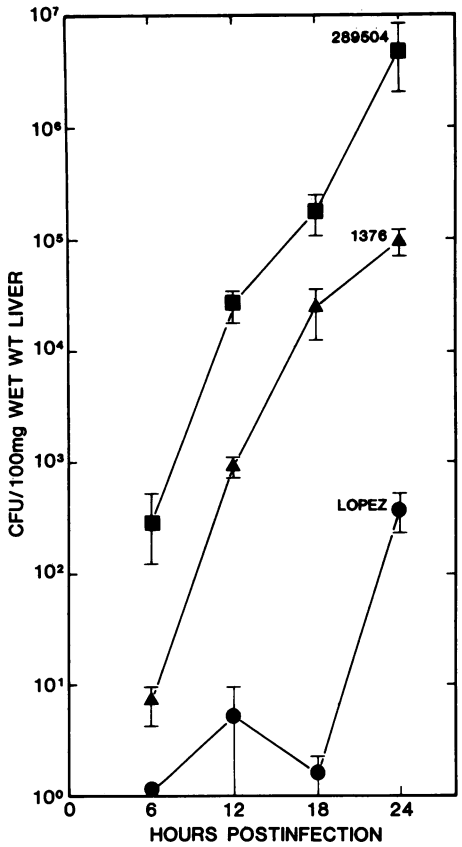

FIG. 1. Time course of invasion of *C. jejuni* 289504, 1376, and LOPEZ into the liver of 11-day-old Hy-Line Variety SC chicken embryos. Embryos were inoculated chorioallantoically with 2×10^7 CFU, and the mean CFU present in embryonic livers was followed with time. Each point represents the mean ± the standard error of two separate experiments in which five embryos were used per group.

TABLE 4. Virulence of *C. jejuni* LOPEZ, 1376, and 289504 for 11-day-old Hy-Line Variety SC chicken embryos inoculated intravenously

Strain	Time (h) postinoculation	LD_{50}	
		Live cells	Heat-killed cells
LOPEZ	24	9.9×10^5	6.8×10^6
	48	2.4×10^5	6.8×10^6
	72	4.7×10^4	6.8×10^6
1376	24	3.9×10^3	3.2×10^7
	48	3.9×10^1	3.2×10^7
	72	3.9×10^1	3.2×10^7
289504	24	2.5×10^3	2.2×10^7
	48	2.5×10^0	2.2×10^7
	72	2.5×10^0	2.2×10^7

The results presented in Table 3 and Fig. 1 strongly suggest that lethality is directly related to the invasiveness of the organism or its ability to survive in vivo or both. It should be recalled that embryos infected with a dose of 2×10^7 CFU of any of the three strains will survive the infection for 24 h. However, by 48 to 72 h postinfection, differences in virulence between the three strains (as measured by embryo deaths) are readily apparent. Embryos inoculated on the CAM with either strain 1376 or 289594 are invariably dead within 72 h, whereas those infected with strain LOPEZ are still viable for up to 6 days postinfection. These observations, taken with the data presented above, seem to indicate that strains 1376 and 289504 are virulent because they can successfully invade the CAM and proliferate within embryonic tissues. It is not possible to determine from these experiments, however, why strain LOPEZ is poorly virulent. This strain may be unable to invade across the CAM and reach the embryo; alternatively, it may be invasive but may lack virulence factors which allow it to survive and multiply within the embryo. To distinguish between these two possibilities, additional experiments were carried out with embryos inoculated intravenously.

Virulence of three strains of *C. jejuni* after intravenous inoculation. Graded doses of either live or heat-killed cells of strains LOPEZ, 1376, and 289504 were inoculated intravenously into 11-day-old chicken embryos. Table 4 summarizes the LD_{50}s calculated at 24, 48, and 72 h postinfection. When live cells were administered, the LD_{50} of each strain was lower than that after chorioallantoic inoculation, although the same relative virulence of the strains was maintained. For strain LOPEZ, the LD_{50} at 72 h was decreased by approximately 4 logs, whereas for strains 1376 and 289504 it was decreased approximately 2 logs. These results clearly indicate that strain LOPEZ is less invasive than the other two strains, since once introduced into the embryo it can survive and proliferate. The ability to invade, however, does not appear to be the only factor involved in the virulence of the other two strains. Strains 1376 and 289504, with their unusually low LD_{50}s at 72 h, must have unidentified virulence factors which strain LOPEZ, especially, does not possess.

The LD_{50} for heat-killed cells was approximately the same for each of the three strains and did not vary with time postinfection. Thus, the differences in virulence between the strains cannot be directly attributed to differences in the inherent toxicity of the campylobacter cell wall.

TABLE 5. Effect of motility on virulence of *C. jejuni* 1376 for 11-day-old Hy-Line Variety SC chicken embryos inoculated chorioallantoically

Cell type inoculated[a]	LD_{50} at 72 h (no. of embryos per dose)	Mean CFU in embryonic liver[b] at 24 h ± SEM
Wild type	8.2×10^3 (15)	$6.1 \times 10^5 \pm 4.3 \times 10^5$
Highly motile variant	2.9×10^3 (9)	$4.9 \times 10^5 \pm 2.1 \times 10^5$
Nonmotile variant	4.6×10^2 (9)	$6.6 \times 10^5 \pm 5.6 \times 10^5$

[a] The wild-type strain consisted of a mixture of both motile and nonmotile colony types when plated in soft agar. Highly motile and nonmotile variants were selected from the wild-type strain as described in the text.

[b] Mean CFU per 100 mg (wet weight) of liver. Five livers were cultured for each cell-type.

TABLE 6. Percentage of motile and nonmotile organisms of *C. jejuni* 1376 present in the inoculum and in livers of 11-day-old Hy-Line Variety SC chicken embryos infected 24 h previously

Cell type inoculated	% Highly motile in:		% Motile in:		% Nonmotile in:	
	Inoculum	Livers	Inoculum	Livers	Inoculum	Livers
Wild type	39	58	26	24	35	18
Highly motile variant	100	100	0	0	0	0
Nonmotile variant	0	0	0	0	100	100

The LD_{50} for heat-killed cells was approximately the same for each of the three strains and did not vary with time postinfection. Thus, the differences in virulence between the strains cannot be directly attributed to differences in the inherent toxicity of the campylobacter cell wall.

Effect of motility on virulence of *C. jejuni* 1376. It has been established that motility plays a significant role in the virulence of certain enteric pathogens (1, 8, 19, 23, 50, 52). There are reports that this is true also for *C. jejuni* (33, 35). Experiments were carried out to determine the effect of motility on the virulence of *C. jejuni* 1376 for chicken embryos. The virulence of the wild-type organism (which consisted of a mixture of motile and nonmotile colony types when plated in soft agar) was compared with that of motile and nonmotile variants selected from the wild-type strain. Eleven-day-old chicken embryos were inoculated chorioallantoically with graded doses of bacteria, and LD_{50}s were calculated after 72 h. The mean number of CFU present in embryonic livers was also determined for groups of embryos infected with a single dose of 2×10^7 organisms. The results of these experiments are presented in Table 5.

There is no strong indication that motility plays a role in the virulence of this strain for chicken embryos as evidenced by the small variation seen in the LD_{50}s. Nor was there a statistically significant difference in the number of CFU recovered from livers of embryos at 24 h postinfection. Additional studies were performed to determine whether reversion or selection of motile and nonmotile variants occurred in vivo. Table 6 presents the results obtained when samples from the inoculum (obtained just before infection) and from liver homogenates (obtained 24 h after infection) were cultured in soft agar pour plates. The phenotype of each culture was determined by observing at least 1,000 CFU per sample. Both motile and nonmotile colony types were recovered in the samples when wild-type organisms were used for infection. The motility characteristics of both variants, likewise, remained unaltered after passage through the embryonic liver. These observations strengthen the concept that motility plays no role in the invasion of chicken embryos by this strain.

DISCUSSION

A persistent problem with studies of campylobacter pathogenesis has been the lack of a suitable animal model which mimics human disease under controlled laboratory conditions. In the absence of such a model, a variety of in vitro and in vivo systems have been employed to study mechanisms of disease production. The studies reported here confirm the usefulness of the chicken embryo as an in vivo model for studying aspects of pathogenesis which relate to invasion.

Human isolates of *C. jejuni* and *C. coli* exhibit striking differences in virulence when inoculated chorioallantoically into 11-day-old embryos. These differences are manifested both by the times of death and by LD_{50}s calculated at 72 h. The number of CFUs culturable from embryonic livers at 24 h directly correlates with the virulence of the strain administered. Since the appearance of campylobacters in the liver necessitates that they successfully penetrate the CAM, virulence in this system reflects strain invasiveness. This interpretation is further substantiated by data obtained when strains of *C. jejuni* were inoculated into embryos via the intravenous route. One of the strains tested, which was poorly virulent after chorioallantoic inoculation, was able to survive and multiply when introduced directly into the embryonic circulation. The two other strains tested, however, which were highly virulent when inoculated chorioallantoically, also exhibited decreased LD_{50}s after intravenous inoculation. These observations indicate that invasiveness alone cannot account for the entire spectrum of virulence displayed by *C. jejuni* strains in this model system.

There appears to be no relationship between the virulence of strains for chicken embryos and their carriage of plasmid DNA. Genetic analysis of some enteric pathogens has shown that plasmids are required for full expression of virulence. Large plasmids have been directly associated with invasiveness in strains of *Salmonella typhimurium* (24), *Shigella* spp. (43, 44), *Yersinia enterocolitica* (54), and *Escherichia coli* (42). Although several of the campylobacter strains examined in this study carried large-molecular-weight plasmids, the most virulent strains tested (those with LD_{50}s of $<10^4$) were devoid of detectable plasmid DNA. Thus, virulence in this model system appears to be chromosomally mediated. These findings agree with those of Taylor and Bryner (46), who reported that plasmid carriage did not correlate with the ability of *C. jejuni* and *C. coli* strains to produce abortions in the pregnant guinea pig model.

We can only speculate about the factors which virulent strains possess that enable them to invade the CAM and survive in vivo. Although 11-day-old chicken embryos are incapable of mounting immune responses, they do possess phagocytic capabilities (25, 32). Thus, successful survival in vivo requires that the campylobacters be able to resist destruction by phagocytes. Virulent strains must also be able to acquire growth-essential iron to proliferate within embryonic tissues. Although the mechanisms of invasion are poorly understood, invasiveness in some enteric bacteria has been linked to the possession of unique O-antigen determinants, outer membrane proteins, and iron transport systems (16).

Studies from this laboratory on campylobacter iron transport have demonstrated that some strains of *C. jejuni* can produce siderophorelike compounds in vitro (11). However, the results presented here indicate that not every strain which produces a siderophore in vitro is virulent for chicken embryos. In fact, the two most virulent strains tested in this study (289504 and 303955) did not appear to synthesize iron-chelating compounds. It is possible that these strains produce siderophores in vivo and that we were unable to detect their production in vitro. Alternatively, strains which are unable to synthesize siderophores may have other methods for acquiring iron in vivo.

Experiments are currently in progress to determine whether virulence for the embryos can be associated with one or more components of the campylobacter cell wall or with the production of toxin by these strains. Preliminary evidence indicates that differences in lethality cannot be related to differences in inherent toxicity of campylobacter cells. Heat-killed cells of a highly virulent and a relatively avirulent strain were equally lethal when injected intravenously into chicken embryos. Thus, although endotoxin or other toxic components of the cell wall may ultimately be responsible for death of the embryos once the number of bacteria reaches 10^6 to 10^7 organisms, additional factors are necessary to account for the virulence of some strains. It is possible that virulent strains possess unique O-antigenic determinants or outer membrane proteins which render them antiphagocytic in vivo.

The results of these studies seem to indicate that motility plays little role in the invasive process within the chicken embryo. Similar LD_{50}s were obtained when embryos were infected chorioallantoically with either wild-type, motile, or nonmotile cells of *C. jejuni* 1376. Approximately the same number of organisms of each cell type was also recovered from embryonic livers 24 h after infection. In contrast to the observations of Caldwell et al. (6), the nonmotile variant did not appear to revert to a motile phenotype in vivo. Neither was there positive selection for motile forms or reversion of nonmotile forms when wild-type cells were administered. The lack of correlation between motility and virulence in this model should not necessarily be extended to other systems, where motility may be important for adherence and colonization. Since the pathogenicity of campylobacters is no doubt determined by a multitude of virulence factors, motility may play an important role in the infectious events which precede invasion.

The relationship between the virulence of campylobacter strains for chicken embryos and their pathogenicity for humans is as yet only conjectural. Nevertheless, there are obvious experiments that relate to invasion which can now be undertaken with this model system.

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