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Response of the chick embryo to live and heat-killed *Campylobacter jejuni* injected into the yolk sac

A. G. CLARK AND D. H. BUESCHKENS

Department of Microbiology, University of Toronto, 150 College Street, Toronto, Ontario, Canada M5S 1A8

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SUMMARY

Graded doses of live and heat-killed cells of *Campylobacter jejuni* were injected into the yolk-sac of 5-day-old chick embryos, and the 50% lethal dose (LD_{50}) was determined 7 days later. A strain dependent virulence was seen. In the diluted series of cultures the LD_{50} values for live campylobacter ranged from 10^6 c.f.u. beyond the last dilution showing growth, that is to less than one organism per embryo. When the 22 strains were tested as heat-killed cells, the chick embryo LD_{50} values retained the same relative order of toxicity obtained with viable cells, but the LD_{50} values were increased by +1 to +4 log units. Heat-killed cells from strains known to be invasive, but non-toxigenic, were still lethal for the embryos, suggesting that viability was not solely necessary for virulence. Semi-pure lipopolysaccharide from a non-virulent strain of *C. jejuni* was not toxic to the embryos, but semi-pure and ultracentrifuge-purified lipopolysaccharide from the most lethal campylobacter strains gave LD_{50} values in the order of 3·0 μg lipopolysaccharide per ml (0·6 μg per embryo) in the yolk-sac assay. No relationship between serotype and lethality was seen. Injection into the yolk-sac appears to be an easy, rapid and reproducible *in vivo* assay of the virulence of *C. jejuni*.

INTRODUCTION

Campylobacter jejuni is a major cause of acute enterocolitis, but there is no one laboratory animal in which all of the clinical symptoms of human campylobacteriosis can be reproduced. The two clinically severe presentations are described as bloody diarrhoea and watery (secretory) diarrhoea which, respectively, suggest invasive and toxigenic virulence factors. Laboratory evidence supports the existence of a variety of putative virulence factors but only a toxin, immunologically related to *Escherichia coli* LT and cholera enterotoxin, has been isolated in a semi-pure form (1). Neither the cytotoxin (2) nor the invasive factor have been characterized, although the outer membrane proteins and lipopolysaccharides are under investigation for their possible implication as virulence factors (3–5). Despite the large number of *C. jejuni* isolated and reported in the literature, few strains have been assayed for invasiveness. Toxigenicity assays were carried out on isolates from Mexico and the USA by Klipstein and co-workers (6). Subsequently this group showed that of 40 strains obtained from one

asymptomatic child and 39 sequentially admitted human cases of campylobacter-related diarrhoea, 14 strains produced enterotoxin, 13 were invasive, 2 were both toxigenic and invasive, but 11 strains were neither toxigenic nor invasive (7). Other virulence factors, therefore, must exist. Johnson and Lior similarly found that not all patient isolates produced the detectable cytotoxic or cytotonic virulence factors (2).

Originally Ruiz-Palacios and co-workers (8) had fed *C. jejuni* to 3-day-old chicks and, depending on the dose, induced diarrhoea in up to 88% of the birds. Welkos (9) had fed *C. jejuni* to chicks that were less than 3 days old and all subsequently passed blood in their faeces. Previously, Davidson and Solomon (10) had injected *C. jejuni* into chick embryos at different stages of development and observed an increasing resistance to embryo invasion after 11 days of egg incubation. The embryos became totally refractory to infection if older than 17 days due to the development of the immune system. This work has been extended by Field and colleagues (11) who showed that when campylobacters were injected onto the chorioallantoic membrane of 11-day-old chicken embryos, the LD_{50} values were inversely proportional to the number of campylobacters isolated from the liver. That is, the decreased LD_{50} equated with an increased invasiveness.

In the past we examined the effect of *C. jejuni* on the hatchability of fertile chicken eggs and the growth of the campylobacters in the egg (12, 13). Most of the published work with chick embryos has followed the commonly used, and destructive, chorioallantoic route, injecting through a hole cut in the shell onto the membrane or into the developing veinous system. As an alternative we infected embryos by introducing the campylobacters into the undamaged egg by temperature differential just before the start of egg incubation (12). In this paper we report the effect on embryo viability of injecting live or heat-killed *C. jejuni* directly into the yolk sac of 5-day-old chick embryos.

MATERIALS AND METHODS

Bacteria and growth conditions. The strains listed in Table 1 as PC 72, TGH 9585, CEPA 4C, J 32, SC 138, MK 5, MK 155, MK 7, Van A5 and MK 307 were received from J. L. Penner, Department of Microbiology, University of Toronto; F. A. Klipstein, University of Rochester Medical Centre, Rochester, New York supplied strains C006, INN 73-83, Case and V48, while strains 81-176 and 81-159 were given by M. J. Blaser, Veterans Administration Medical Center, Denver, Colorado. Strains DCT, CN 2 and CN 9 were isolated from retail chicken by ourselves, while SC138 (J. Prescott, Ontario Veterinary College, Guelph, Ontario, Canada) was from a poultry slaughterhouse and PC72 was of bovine origin. The strains CEPA 3C, CEPA 4C, and INN 73-83 had originally been isolated by G. M. Ruiz-Palacios, Instituto National de la Nutricion, Tlalpan, Mexico. Strains INN 73-83, 79-102, C006, Case and V48 were among those used by Klipstein and co-workers (6) to demonstrate toxigenesis or invasiveness in *C. jejuni*.

C. jejuni strains were grown routinely at 42 °C under a gas phase of $N_2:CO_2:O_2$ in the ratio 85:10:5 on 7% horse blood agar (Columbia agar base, Gibco, Canada) with the reductants of George and co-workers (14). After incubation for 48 h the cells were washed off the plates with 0·1% peptone water (Oxoid Canada Ltd),

washed by centrifugation in the same medium, and suspended to OD_{650} equivalent to 10^8 c.f.u. per ml. A viable count was made in triplicate on blood agar using 0·1 ml of dilution on a spread-plate. When heat-killed cells were required the same procedure was repeated and, after the viable count had been made, the suspension was heated at 60 °C for 20 min. The sample was cooled and a second viable count was made to confirm sterility. Viable and heat-killed cells were serially tenfold diluted in 0·1% peptone water and 0·2 ml of each dilution was injected into the yolk-sac of candled, 5-day-old, embryonated eggs in series of fives. Five control eggs received 0·2 ml peptone water per organism or extract under test.

Egg inoculation and LD_{50} assay. The fertile eggs were bought in lots of 360–420 from one registered hatchery and were set within 24 h of being laid. The eggs were serially numbered on receipt and randomized in incubators as described elsewhere (12). After 5 days of incubation the eggs were candled and the infertiles were counted and discarded. The fertile eggs were grouped in series of fives for the LD_{50} assay. Standing with the air sac (blunt end) uppermost, the top of each egg was swabbed with 2% ethanolic iodine and allowed to air dry. A sterile diamond-pointed or carbide glass scriber was used to give a single rapid blow to the shell. This caused sufficient damage to allow a 27-gauge, 38 mm (1·5 inch) needle (Becton-Dickinson, USA) to be vertically inserted into the egg to a depth of 32 mm (1·25 inches). Each egg had received 0·2 ml of bacterial suspension, or for the controls 0·2 ml 0·1% peptone water, and the hole in the shell was closed with clear, fast drying glue (Lepage Household Cement). Incubation with turning was continued for a further 7 days, with each egg being candled daily. The eggs were removed from the incubators and asceptically broken so that the stage of development of each embryo was noted. Embryos at day 11 or 12 of development were scored together to avoid subjective bias because the differences between day 11 and 12 development is difficult to identify in some cases. Each stage of development was scored and the LD_{50} was calculated by the method of Reed and Muench (15) based upon the serial dilutions used. All embryos which had died were cultured by streaking a yolk sample on blood agar and incubating aerobically at 37 °C for 48 h. Eggs giving bacterial growth which was not *C. jejuni* were discounted from the calculations. The data were rejected if no one batch of five eggs in the dilution series of treatments showed greater than 50% death. In most cases both 0 and 100% effects were obtained, and only tests in which the 50% end point was bracketed are included in the tabulations.

In order to evaluate the possibility of cross contamination between sets of eggs via the humidity water in the incubators, the water from the bottom of each incubator was plated in triplicate on Skirrow-type blood agar plus antibiotics at the end of each egg incubation (12). After 48 h at 42 °C the plates were examined for *C. jejuni*; none were detected.

Lipopolysaccharide extraction. Fifty blood agar plates were seeded for each strain and, after 48 h incubation as described above, were harvested into sterile, pyrogen-free saline containing 1·0% formaldehyde. The cells were pelleted at 4000 ***g*** for 20 min, washed twice with sterile saline, and the pellet transferred to a phenol–water mixture in the ratio 2 gm wet cells per 70 ml phenol–water. The phenol–water mixture contained equal volumes pyrogen-free water and 90% phenol. The cell mixture was homogenized for 5 min in a blender and stirred for

Table 1. *Lethality of* Campylobacter jejuni *inoculated into the yolk sac of 5-day-old chicken embryos*

Strain	Serotype (Penner)	Source*	LD_{50} at 12 days: Live bacteria (c.f.u. per embryo)†	LD_{50} at 12 days: Heat-killed bacteria (equivalent c.f.u. per embryo)
C006	4	Human	$4{\cdot}9 \times 10^{6}$	$> 10^{9}$
PC 72	2	Calf	$4{\cdot}4 \times 10^{4}$	$6{\cdot}5 \times 10^{6}$
TGH 9585	NT‡	Human	$3{\cdot}8 \times 10^{4}$	$2{\cdot}8 \times 10^{9}$
CEPA 4C	3	Human	$2{\cdot}2 \times 10^{4}$	$3{\cdot}6 \times 10^{6}$
81-159	13:16:50	Human (poultry)	$1{\cdot}6 \times 10^{3}$	$1{\cdot}0 \times 10^{5}$
B2758/81	1	Human (water)	$7{\cdot}5 \times 10^{2}$	$8{\cdot}4 \times 10^{6}$
CN 2	10	Chicken	$3{\cdot}2 \times 10^{2}$	$1{\cdot}6 \times 10^{6}$
81-176	23:36	Human (cattle)	$2{\cdot}6 \times 10^{2}$	$3{\cdot}1 \times 10^{7}$
J 32	6. 7	Human	$2{\cdot}1 \times 10^{2}$	$1{\cdot}8 \times 10^{5}$
SC 138	3	Chicken slaughterhouse	$1{\cdot}3 \times 10^{2}$	$3{\cdot}1 \times 10^{4}$
MK 5	1	Human	$6{\cdot}0 \times 10^{1}$	$1{\cdot}3 \times 10^{8}$
CN 9	8:23:36	Chicken	$2{\cdot}6 \times 10^{1}$	$2{\cdot}8 \times 10^{4}$
DCT	1	Chicken	$2{\cdot}4 \times 10^{1}$	$1{\cdot}0 \times 10^{7}$
MK 155	11	Human	$1{\cdot}3 \times 10^{1}$	$4{\cdot}7 \times 10^{6}$
INN 73-83	3	Human	$2{\cdot}1 \times 10^{1}$	$3{\cdot}1 \times 10^{2}$
MK 7	4	Human	$8{\cdot}7 \times 10^{0}$	$3{\cdot}7 \times 10^{4}$
79-102	2	Human	$5{\cdot}4 \times 10^{-1}$	$3{\cdot}4 \times 10^{0}$
Van A5	43	Human	$3{\cdot}4 \times 10^{-1}$	$1{\cdot}1 \times 10^{1}$
V48	NT	Human	$1{\cdot}1 \times 10^{-1}$	$2{\cdot}4 \times 10^{2}$
MK 307	50	Human	$8{\cdot}6 \times 10^{-2}$	$6{\cdot}0 \times 10^{2}$
Case	NT	Human	$7{\cdot}6 \times 10^{-2}$	$2{\cdot}6 \times 10^{1}$
CEPA 3C	3	Human	$6{\cdot}0 \times 10^{-2}$	$1{\cdot}9 \times 10^{0}$

* Source of isolation, source of infection in parentheses where known.
† 0·2 ml injected per embryo.
‡ NT, not typable.

20 min at 65 °C then the suspension was cooled to 4 °C. After centrifuging at 12000 g for 30 min, the upper aqueous layer was removed and dialysed against several changes of deionized water until free of phenol. The dialysed lipopolysaccharide was lyophilized until used, when the powder was dissolved in pyrogen-free distilled water and serially diluted before injection (0·2 ml) into the yolk-sac of 5-day-old chicks. Samples of ultracentrifuge-purified lipopolysaccharide were obtained from J. L. Penner. These samples had been examined in electrophoretic and immunochemical analyses (16). The samples had been shown to contain no protein as measured by the Bio-Rad Protein assay (Bio-Rad Laboratories, Canada) and gave no Coomassie Blue-positive bands in SDS–PAGE. However, the Bio-Rad assay has a minimum threshold for detection of 3% by weight of the sample assayed.

RESULTS

Live bacteria. Table 1 is constructed with downward decreasing LD_{50} values for live bacteria. The strains showed marked variation in their lethality when

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Table 2. *Lethality of lipopolysaccharide from* Campylobacter jejuni *for 5-day-old chicken embryos*

	LD_{50} at 12 days		
Strain*	Heat-killed bacteria (equivalent c.f.u.)	μg LPS per embryo†	Virulence factors‡
C006	$> 10^9$	$> 10^4$	N
CN 9	$2{\cdot}8 \times 10^4$	$> 10^4$	U
INN 73-83	$3{\cdot}1 \times 10^2$	$7{\cdot}9 \times 10^2$	I, T
MK 7	$3{\cdot}7 \times 10^4$	$2{\cdot}3 \times 10^3$	U
79-102	$3{\cdot}4 \times 10^0$	$7{\cdot}9 \times 10^{-1}$	I
Van A5	$1{\cdot}1 \times 10^1$	$3{\cdot}7 \times 10^1$	U
V 48	$2{\cdot}4 \times 10^2$	$1{\cdot}1 \times 10^0$	T
MK 307	$6{\cdot}0 \times 10^2$	$2{\cdot}7 \times 10^1$	U
Case	$2{\cdot}6 \times 10^1$	$6{\cdot}3 \times 10^{-1}$	I
CEPA 3C	$1{\cdot}9 \times 10^0$	$5{\cdot}0 \times 10^0$	t

* Strains appear in same decreasing order as in Table 1. The heat-killed results are repeated for comparison.

† 0·2 ml injected per embryo.

‡ N, neither T or I; T, toxigenic; I, invasive; all as described in references 6 and 7; U, unknown; t, toxigenic (personal communication from G. Ruiz-Palacios to J. L. Penner).

introduced directly into the yolk-sac, and this lethality did not correlate with the Penner serotype of the injected bacteria. Parallel control inoculations gave no embryo death or egg contamination due to the inoculation procedure. Different batches of eggs did not affect the LD_{50} values of the strains in repeated tests, the maximum LD_{50} variation being one log value. The last five strains listed in Table 1 gave repeated LD_{50} values of less than one viable organism. Previous studies on hatchability had shown that strains V48, Case and CEPA 3C markedly decreased the hatchability of eggs when passively introduced into the eggs by temperature differential (12), while strains SC138, DCT and CN 9 had no such effect on hatchability (Clark and Bueschkens, unpublished data).

Heat killed bacteria. When the same 22 strains were tested in the LD_{50} assay with heat-killed bacterial suspensions, the live strains seen at the extremes of the LD_{50} values in Table 1 retained their general positions of lethality. The six most lethal live strains remained the most lethal when tested as heat-killed suspensions, but the LD_{50} values now increased by plus one to plus four logarithmic units. All the other strains also required higher doses of heat-killed bacteria for 50 % lethality, but none was rendered as avirulent as the negative control strain C006.

Lipopolysaccharides. Lipopolysaccharide was prepared from 10 of the 22 strains listed in Table 1, and the LD_{50} values per embryo are listed in Table 2. Strains CN 9 and C006 gave LD_{50} values beyond 10 mg per embryo, while the LD_{50} value for strain Case was 0·63 μg per embryo. Irrespective of the reported invasiveness or toxicity of the strains, the phenol–water extracted LPS showed marked lethality. LPS purified by ultracentrifugation from strains Van A5 and MK 307 had been shown by immunoblotting to have high M_r LPS (16). The LD_{50} values for phenol–water extracted LPS from strains Van A5 and MK 307 were $3{\cdot}7 \times 10^1$ and $2{\cdot}7 \times 10^1$ μg LPS per embryo respectively, as seen in Table 2, but for LPS purified

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by ultracentrifugation these values were 3·3 and $4{\cdot}1 \times 10^{0}$ μg per embryo respectively. There was thus a small diluting effect caused by contaminants in the phenol–water material, and these contaminants did not enhance the lethality of the LPS.

DISCUSSION

In this study it has been shown that live and heat-killed *C. jejuni* are capable of killing 5-day-old chick embryos. The lethality varied with the strain, but agrees with other evidence for the virulence of some of the strains. The original work of Klipstein's group demonstrated that invasiveness and toxigenicity could be assayed by ELISA tests. The strains they used to develop their assay included INN 73-83, 79-102, V48, Case and C006. Strain C006 was their negative control. In the present work this latter strain was the least pathogenic strain employed, while the other strains occupied the other extreme of the LD_{50} values. In passive inoculation of fertile eggs at day zero of incubation, we have previously shown that strains Case, V48, 79-102 and INN 73-83 can decrease the hatchability of fertile eggs from the routine 70–80% to less than 40% (Clark and Bueschkens, unpublished data). On the other hand, strains C006, CN2 and CN9 had no influence on hatchability. The inoculation in these hatchability experiments was via the non-invasive route of temperature differential in which the inoculum size was dependent on the physical properties of the egg shell, but was in the range of 10^3 to 10^6 c.f.u. per egg. In the LD_{50} assays reported here, the embryo mortality was reproducible in repeated assays, and the initial failures to obtain viable cell LD_{50} values bracketed by zero mortality for the six strains 79-102 to CEPA 3C (Table 1) were overcome by diluting the cultures past extinction of the viable count. There are two possible explanations for this: either the preparation of the dilutions liberated a toxic compound from the cells, or the preparations contained cells which were not detected by culture methods. Viable, but non-culturable, coccoid cells have been shown to exist in environmental vibrio cultures (17). Viable, but non-culturable, forms of *C. jejuni* have been demonstrated in water implicated as sources of farm and human infection (18). *C. jejuni* cultures can also lose the vibrioid shape and develop into round forms which have been suggested to be non-culturable. However, no such coccoid forms were seen in the preparations used.

Filtration of the culture suspensions through 0·22 μm filters did not remove the lethal effect of the diluted filtrates for 5-day-old embryos when compared with the parent culture. The nature of the lethal principle has not been identified, nor is it certain that the effect is due to only one agent, but the LD_{50} value of the LPS of strain CEPA 3C is decreased in the presence of homologous antisera to the whole cell and also by antisera to the cholera B subunit (Clark, unpublished data). Certainly, some strains have been shown to possess more than one virulence factor. Strains INN 73-83, V48 and CEPA 3C are known to be toxigenic, while strains INN 73-83, 79-102 and Case have invasive properties. When these strains were assayed as heat-killed cells in the embryos, the LD_{50} values increased by plus one to four logarithmic units. The lethal agent(s) was thus partly sensitive to 60 °C for 20 min. More importantly, those cells which were purely invasive (79-102 and Case) remained lethal for 5-day embryos when injected in the heat-killed

form. Viability was therefore not necessary for the virulence of these strains, which suggests other factors must act. In general, all strains when tested as heat-killed cells showed decreased virulence (increased LD_{50} value); strains MK 155, DCT, MK 5, 81-176 and TGH 9585 showed the greatest increase in the LD_{50} value. None of these lethal effects corresponded to the serotype of the strains; for instance Penner serotypes 3 and 4 appear throughout Table 1.

The LD_{50} values for LPS alone paralleled those for heat-killed cells (Table 2). These LPS preparations contained less than 3% protein (the threshold of detection in the BioRad assay). Preston and Penner (16) showed that while most of their strains possessed only low M_r LPS in SDS–PAGE, some strains possessed both low and a series of high M_r LPS components. Silver staining of SDS–PAGE gels revealed only low M_r LPS as has been found by others (3, 19, 20), but by immunoblotting, a third of these serotyped strains were shown to possess structures characteristic of LPS with O-sidechains of varying length (16). However, strains with only low M_r LPS still showed serospecificity demonstrable by immunoblot tests as well as by passive haemaglutination titrations. Thus it seems that serospecificity resides in both high and low M_r LPS. The composition of LPS from *C. jejuni* and its role in antigenicity and virulence are not yet resolved. Naess and Hofstad (21) found that acetic acid-degraded LPS from strains of *C. jejuni* fell into two classes when examined by gel filtration. Some strains had LPSs consisting only of lipid A and core sugars, while others had LPSs with 10–25 hexoses per three heptoses.

Field and co-workers (11) have examined the chicken embryo as a model for invasion when campylobacter is injected onto the chorioallantoic membrane of 11-day-old fertile eggs. The LD_{50} 72 h later showed a six logarithmic variation between strains. The number of campylobacters culturable from the liver was inversely related to the LD_{50} dose for the strain. This virulence was not dependent on motility. When graded doses of either live or heat-killed cells from three selected cultures were intravenously injected by Field and co-workers (11), there was an increase of LD_{50} value of +1 to +6 logarithmic units; the largest change occurring for the most invasive strain tested. They concluded that endotoxin was not a factor in embryo death unless 10^6 to 10^7 cells were present (11). This they based on the comparison of the three strains, one of which was highly virulent, while another was relatively avirulent when alive, yet they each gave the same LD_{50} value when injected intravenously as heat-killed cells into chicken embryos. Stuart-Tull and co-workers (22), however, concluded that for HAM/ICR mice, the intraperitoneal injection of heat-killed *C. jejuni* resulted in death due to endotoxin.

Twenty years ago there was considerable interest in the response of chicken embryos to endotoxin (23–27). When Finkelstein examined the chick as an alternative test model for cholera endotoxin, he found that the route of injection and the age of the embryo were important in evaluating susceptibility (27, 28). He injected 11-day embryos intravenously with endotoxin and obtained LD_{50} values of 0·005–0·14 μg per embryo for *Vibrio cholerae*. Understandably, endotoxin was less effective when administered onto the chorioallantoic membrane than when injected into the veinous system. Originally, Smith and Thomas (25) had shown intravenous injection of meningococcal LPS to be useful in chick embryo assays only when the embryo was 8–12 days old. Finkelstein and Ransom (29) detected

an increasing refractoriness to viable *Vibrio cholerae* or *Shigella flexneri* endotoxin between 13 and 15 days of embryo age; again the results were read early (24 h), but in this case after allantoic challenge. The yolk-sac route was found to cause more rapid death in 11-day-old chicks than was caused by allantoic inoculation of live *V. cholerae* (28). There are thus a variety of inoculation routes and embryo ages reported, although a current standard for LPS assays is the chorioallantoic route (laid on or injected intravenously) for embryos 7–10 days old. This is a more tedious method than simple yolk-sac inoculation and has a higher chance of contamination. Further, the egg cannot be incubated with egg turning which is a critical factor in embryo development.

The method reported in this paper enables one to examine the virulence of many campylobacter strains rapidly under conditions in which *C. jejuni* is known to grow (12, 13). There is the obvious proviso that the eggs should be from a constant source and should not have been fumigated at the hatchery. At present we do not know what contribution is made by the possible release of the known cholera-like enterotoxin of *C. jejuni* to embryo lethality, nor will the role of LPS in virulence be clarified until a chemical analysis is made of the component compounds.

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