Study of the Infectivity of Saline-Stored *Campylobacter jejuni* for Day-Old Chicks

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The culturability of three *Campylobacter jejuni* strains and their infectivity for day-old chicks were assessed following storage of the strains in saline. The potential for colonization of chicks was weakened during the storage period and terminated 3 to 4 weeks before the strains became nonculturable. The results from this study suggest that the role of starved and aged but still culturable campylobacters may be diminutive, but even more, that the role of viable but nonculturable stages in campylobacter epidemiology may be negligible. Even high levels of maternally derived anti-campylobacter outer membrane protein serum antibodies in day-old chicks did not protect the chicks from campylobacter colonization.

Worldwide, campylobacteriosis is one of the most common enteric diseases. One of the many reservoirs of *Campylobacter* infection is poultry. Some investigations have indicated that very low doses of thermophilic *Campylobacter* cells are sufficient to colonize chicks (1, 17, 26, 27, 32). However, the sources of *Campylobacter* infection in broiler flocks have usually remained undetermined due to the ubiquitous prevalence of the two most common *Campylobacter* species, *Campylobacter jejuni* and *Campylobacter coli*. According to the results of survival experiments with *Campylobacter* spp. (3, 16, 30), the strains can survive for prolonged periods in laboratories (24). *Campylobacter* cells also are known to survive for an undetermined period in culturable form in the environment, most often in water (15, 24). However, the role of a *Campylobacter*-contaminated environment as a source of infection in broiler flocks is unclear, since the ability of *Campylobacter* cells to remain infective under environmental conditions has been little investigated. Furthermore, it has been proposed that *Campylobacter* cells that do survive in the environment are in a viable but nonculturable (VBNC) stage (24). The epidemiological significance of VBNC-stage *Campylobacter* cells from environmental waters is not clear-cut. On one hand, *Campylobacter*-contaminated water has been identified as an important risk factor for campylobacteriosis in some risk analyses (2, 6, 12, 14), and several reports of waterborne outbreaks of campylobacteriosis have been published (7, 21, 22). On the other hand, Newell et al. (19) found that an environmentally isolated *Campylobacter* strain loses most of its virulence after storage in water. Concerning the potential infectivity of VBNC-stage *Campylobacter* strains produced in laboratories, experimental studies also have been contradictory; some have had a negative outcome (8, 17, 31), and others have suggested that *Campylobacter* cells in VBNC stages can colonize chicks (13, 25, 28).

This study investigated the ability of three *C. jejuni* strains to colonize day-old chicks during storage of the strains in saline suspensions. The *C. jejuni* strains used were a broiler strain

(G97-76595, serotype O:27), a human clinical isolate (KI-5133, serotype O:2) (7), and a laboratory-adapted strain (Pen-6 ATCC 43434, serotype O:6), which was obtained from the Penner serotype collection (23). The dose-response relationship and the ability to colonize chicks on days 1, 9, 19, 30, and 39 of storage in saline were assessed. Suspensions of the strains were prepared from 24-h cultures grown on blood agar (blood agar base 2 [Oxoid, Basingstoke, United Kingdom] supplemented with 5% calf blood) by shaking of bacterial material in 0.9% saline at 2 to 4°C. For the dose-response experiments, the suspensions were used within 4 to 6 h. For the storage experiments, the suspensions were kept stationary under aerobic conditions in the dark at 16°C in screw-cap glass bottles. For each experiment, the CFU per milliliter was determined ca. 2 h before and ca. 2 h after each inoculation, and the inoculation dose was calculated as the average of the two CFU-per-milliliter totals. Total cell counts, which were determined by the acridine orange direct-count technique (11), remained constant throughout the experiments in all three suspensions.

Day-of-hatch chicks were obtained from a commercial Danish hatchery. Within 24 h after hatching, the chicks were wing-marked and all chicks were tested to determine whether they carried *Campylobacter*. The chicks were then inoculated with *Campylobacter* cells and housed in sterilized isolators (Montair Andersen, type HM 1500) floored with a grid. The dose-response relationship for each strain was assessed by inoculation of each of five groups of 9-day-old chicks with serial dilutions of the *Campylobacter* suspensions. Chicks were inoculated orally in the crop through a polystyrene tube, and *Campylobacter* colonization was confirmed on day 4 by culturing from a cloacal swab. For the storage experiment, groups of 10 chicks each were inoculated with stored suspension in the crop at days 1, 9, 19, 30, and 39. *Campylobacter* colonization was confirmed by culturing from a cloacal swab on day 6 and from a cecal swab on day 9, except for the experiment launched at day 30, which was prolonged for 16 days to allow more time for colonization to become established. A noninoculated group was included in each experiment as a control. Cecal tissue with the cranial end of the cecal tonsilla was removed from all chicks postmortem for histopathological examination.

Campylobacter cultures from cloacal and cecal swabs were

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produced by direct streaking of the swabs onto modified charcoal cefoperazone deoxycholate agar (blood-free agar base with cefoperazone at a concentration of 32 mg/liter and amphotericin at a concentration of 10 mg/liter) (Oxoid) and incubated microaerobically in 7% O₂-10% CO₂-83% N₂ at 42°C for 48 h. Plates that were culture negative at 48 h were re-inoculated for an additional 48 h. *Campylobacter* sp. identification was performed according to previously described methods (9).

Typing of the *flaA* gene of the inoculation strains and of strains isolated from colonized chicks was performed as an internal control. DNA was isolated from fecal samples from two randomly chosen *Campylobacter*-positive chicks within each experimental group. Genomic *C. jejuni* DNA was purified from a loopful of bacterial culture using the QIAamp DNA minikit (Qiagen, Hilden, Germany). A 1,705-bp fragment of the *C. jejuni flaA* gene was amplified by PCR as described previously (18). Samples of the *flaA* PCR product were subsequently digested with *DdeI* (Life Technologies, Rockville, Md.) and *AluI* (Boehringer GmbH, Mannheim, Germany) separately, and the digests were analyzed by 3% agarose gel electrophoresis with a 100-bp DNA ladder (Life Technologies) as the molecular size marker.

Blood samples for monitoring of maternally derived serum anti-*Campylobacter* antibodies by an enzyme-linked immunosorbent assay (ELISA) procedure were collected from an extra batch of 10 chicks at each experiment. The average antibody response of this batch served as a reflection of the antibody response of all chicks in an experimental round. A *C. jejuni* outer membrane protein (OMP) preparation was produced from formalin-killed broth culture of a newly isolated *C. jejuni* strain (Penner 4-complex,) as described by Tagawa et al. (29). PolySorp microtiter plates (Nunc, Roskilde, Denmark) were coated with OMP antigen in 0.1 M sodium carbonate (pH 9.6) with 1 M NaCl overnight at 4°C and blocked for 15 min with 1% bovine serum albumin (BSA) and 0.05% (vol/vol) Tween 20 (T) in phosphate-buffered saline (PBS). On each plate, sample and reference sera were diluted 1:200 in BSA-T-PBS and were applied in duplicate for 1 h at room temperature, after which horseradish peroxidase-conjugated rabbit antibody to chicken immunoglobulin (AP 162 P; Chemicon) diluted 1:4,000 in BSA-T-PBS was applied for 1 h. The mixture was then incubated with substrate (*o*-phenylenediamine-H₂O₂ in citrate buffer [pH 5.0]) for 20 min. Color development was stopped by adding 0.5 M H₂SO₄, and optical density was read at 490 nm. Four negative and three positive reference sera were included on all plates in order to control day-to-day variation and interplate variation (see Table 2 below). The OMP ELISA was designed so as to give broad-range detection of anti-*C. jejuni* antibodies, irrespective of serotype (B. Mariager, T. Nikopousius, E. M. Nielsen, S. On, B. Nielsen, M. Madsen, and P. Lind, unpublished data).

For histopathological examination, the cecal tissue was placed in 4% formaldehyde which was phosphate buffered (pH 7), embedded in paraffin, cut in 3- μ m sections, mounted on microscope slides (SuperFrost/Plus; Menzel-Glaser, Braunschweig, Germany), and analyzed for the presence of *C. jejuni* by in situ hybridization (20).

Results from the dose-response experiment are shown in Fig. 1. The dose-response relationship for each strain is expressed as a linear regression line calculated with the Mi-

FIG. 1. Doses of *C. jejuni* strains in the dose-response experiments and the corresponding percentage of colonized birds in the experimental groups. The dose-response relationship is displayed as a linear regression line for each series of data. $\blacktriangle$, chicken strain G97-76595; $\square$, the human strain KI-5133; $\bullet$, laboratory strain Pen-6.

crosoft Excel 97 SR-2 computer program (Microsoft Corp., Seattle, Wash.). Table 1 presents results from all inoculated groups from both the dose-response and the storage experiments, together with data on the inoculation material. In the storage experiments, the infectivity dropped between the day 9 and day 19 experiments; the percentage of infected chicks decreased from 100% on day 9 to 0% on day 19 for both the chicken and the human *Campylobacter* strains and from 90 to 10% for the laboratory-adapted strain. This decrease represents a conspicuous weakening of infectivity, since the inoculation doses given at day 19 (Table 1) would have been expected to colonize approximately 80% of the group inoculated with the chicken strain, 40% of the group inoculated with the human strain, and 35% of the group inoculated with the laboratory strain based on the dose-response relationship displayed in Fig. 1. None of the chicks was colonized after day 19, despite the fact that all three *Campylobacter* suspensions maintained culturability until around day 39. Prolongation of the experiment launched at day 30 to permit multiple intestinal passages of *Campylobacter* did not promote colonization, but we realize that the walking grid that floored the isolators was not optimal for that kind of experiment. Investigation of tissue sections from the ceca showed that the *C. jejuni* cells were localized in the lumens of the intestinal crypts. No invasion of the intestinal cells was observed.

Figure 2 shows the results from the typing of the *flaA* gene of the inoculated strains and of isolates obtained from the cloacal or cecal swabs of colonized chicks at day 9 of the experiment. The three strains had clearly distinguishable *flaA* types. The *flaA* types were designated as follows: the Pen-6 strain, *flaA* type A; the KI-5133 strain, *flaA* type B; and the G97-76595 strain, *flaA* type C. No changes in the Fla type were observed throughout the experiment. Thus, the storage in saline and the passage through the chickens had no influence on the stability of the *fla* types obtained with two different enzymes, which demonstrates the applicability of *fla* typing as a method of controlling possible contamination of strains between experimentally infected chickens in short-term studies.

TABLE 1. Colonization of day-old chicks after inoculation with three *C. jejuni* strains in dilution series of 24-h-old bacteria or under storage in saline suspensions at days 1, 9, 19, 30, and 39

Strain (source)	Serotype and <i>fla</i> type	Dose-response experiments			Storage experiments			
		Initial count (CFU/ml) in suspension	Inoculation dose (CFU/ml)	No. of chicks colonized/no. of chicks inoculated	Day of storage	CFU/ml of suspension	Inoculation dose (CFU/ml)	No. of chicks colonized/no. of chicks inoculated
G97-76595 (chicken)	O:27, <i>flaA</i> type C	1.0×10^8	1.0×10^6	9/9	1	1.6×10^8	1.6×10^7	10/10
			1.0×10^4	6/9	9	3.1×10^8	3.1×10^7	10/10
			1.0×10^2	1/9	19	1.0×10^6	1.0×10^5	0/10
			1.0×10^0	0/9	30	2.0×10^2	2.0×10^1	0/10
			1.0×10^{-1}	0/9	39	3.0×10^{-1}	3.0×10^{-2}	0/10
KI-5133 (human)	O:2, <i>flaA</i> type B	8.8×10^7	8.8×10^6	8/9	1	2.2×10^7	2.2×10^6	10/10
			8.8×10^5	9/9	9	2.3×10^7	2.3×10^6	10/10
			8.8×10^3	4/9	19	4.6×10^4	4.6×10^3	0/10
			8.8×10^1	0/9	30	4.3×10^2	4.3×10^1	0/10
			8.8×10^{-1}	0/9	39	9.0×10^{-1}	9.0×10^{-2}	0/10
Pen-6 (laboratory adapted)	O:6, <i>flaA</i> type A	5.7×10^7	5.4×10^5	4/9	1	3.4×10^8	3.4×10^7	7/10
			5.4×10^3	0/9	9	3.7×10^7	3.7×10^6	9/10
			5.4×10^1	0/9	19	2.7×10^6	2.7×10^5	1/10
			5.4×10^{-1}	0/9	30	1.5×10^3	1.5×10^2	0/10
			5.4×10^{-3}	0/9	39	$<1.0 \times 10^{-1}$	$<1.0 \times 10^{-2}$	0/10

For long-term studies, others (10) have found that Fla typing cannot be considered a stable method for monitoring of *Campylobacter* strains, since intragenomic recombination between *flaA* and *flaB* genes may occur with time.

The chicks that were used for the reported study had serum anti-*Campylobacter* OMP antibodies at levels that ranged from negative (no antibodies) to highly positive (Table 2), as evidenced by the ELISA responses of the corresponding batch control animals. This finding probably reflects the condition of Danish broiler flocks at stocking time, since Danish broiler parent flocks are often infected with *Campylobacter*. Surveillance of 16 parent flocks at a hatchery with a high biosecurity

standard revealed no *Campylobacter*-free flocks at all, not even during the cold season (B. Hald and M. Madsen, unpublished data). In the dose-response experiment, the chicks inoculated with the Pen-6 strain had low levels of antibodies, whereas the chicks used for inoculation with the KI-5133 and G97-76595 strains showed high levels of antibodies (Table 2). The reported serological levels do not indicate a potential protective effect for the OMP antibodies, because the groups with high antibody responses in the dose-response experiments were the groups that were colonized by low doses of either the chicken or human strain, whereas the group that needed high doses for colonization with the laboratory strain had OMP antibody levels that were close to the negative level. Thus, we consider it very unlikely that the low levels of antibody that were present in the groups of chickens inoculated with the stored bacteria interfered with establishment of colonization.

The minimal colonization dose (MCD), that is, the dose that would colonize at least one chick in one experimental group, can be extracted from the dose-response relationship displayed in Fig. 1. The calculated MCD was ca. 10 CFU for the chicken strain, ca. 100 CFU for the human strain, and ca. 10,000 CFU

FIG. 2. Fla typing of inoculation strains and strains isolated from infected chicks. The *flaA* gene was amplified by PCR and digested with either (A) *AluI* or (B) *DdeI*. M, molecular size marker (100-bp DNA ladder); lane 1, Pen-6 inoculation strain; lanes 2 and 3, isolates from chickens colonized with the Pen-6 strain; lane 4, KI-5133 inoculation strain; lanes 5 and 6, isolates from chickens colonized with the KI-5133 strain; lane 7, G97-76595 inoculation strain; lanes 8 and 9, isolates from chickens colonized with the G97-76595 strain. Lane numbers refer to the same information in both panels A and B.

TABLE 2. Serum antibody response in OMP-ELISA from day-old chicks (batch controls) at the time of the inoculation experiments

Experimental group of chicks	Strain or day of storage (no. of chicks)	ELISA optical density (median and range) ^a
Dose-response experiments	G97-76595 (9)	1.34 (0.87–1.92)
	KI-5133 (9)	0.92 (0.19–1.34)
	Pen-6 (9)	0.11 (0.06–0.15)
Storage experiments	Day 1 (10)	0.27 (0.16–0.61)
	Day 9 (10)	0.23 (0.10–0.36)
	Day 19 (10)	0.25 (0.13–0.67)
	Day 30 (10)	0.24 (0.12–0.45)
	Day 39 (10)	0.21 (0.08–0.33)

^a Mean optical densities at 490 nm of four negative control sera were 0.060, 0.055, 0.095, and 0.118; and those of three positive sera were 0.45, 1.08, and 1.59.

for the laboratory strain. For the chicken strain, the calculated dose has been confirmed in a recent experiment, in which the MCD obtained was between 2.7 and 27 CFU (B. Hald and M. Madsen, unpublished data).

The results from this study suggest that higher doses of *Campylobacter* strains from nonchicken sources are needed to colonize chicks when compared with doses of *Campylobacter* strains originating in chickens. This is in agreement with other data that point to chicken passage as a way of enhancing the ability of a strain to colonize chicks. Cawthraw et al. (5) have found that the colonization dose of a laboratory-adapted strain, *C. jejuni* 81116, decreased 10,000-fold to a dose of only 40 CFU per chick for maximal cecal colonization after a single chicken passage. Likewise, Stern et al. (27), by repeated chicken passages of a strain that was originally a poorly colonizing human strain, have produced a *C. jejuni* strain that consistently colonizes chickens. Furthermore, Newell et al. (19) suggest that the virulence of clinical strains is induced by the intestinal passage of the host.

According to our results, the infectivity of the *C. jejuni* strains weakened during the storage in saline, whereas the strains maintained normal culturability. None of the three *Campylobacter* suspensions investigated here colonized any of the chicks, even 3 to 4 weeks before loss of culturability. We propose the term "culturable but not infectious" for such populations of *Campylobacter*. Therefore, the results from this study question the epidemiological significance of environmental survivors, even ones that can readily be cultured and, even more, survivors that are viable but cannot be cultured. Newell et al. (19) have also found a weakening of strains from water sources. They found that water strains were less invasive and less cytotoxic to HeLa cells than were clinical strains of the same serotype and biotype. Most recent VBNC infection studies also have had a negative outcome (8, 17, 31), and it is noteworthy that none of those studies determined at which time the infectivity of the examined strains had actually terminated, since the infection experiments were not launched until after nonculturability was ensured. Some other studies claim, however, that the *Campylobacter* cells in the VBNC stage from aqueous suspensions can be resuscitated by intestinal passage (13, 28). According to our results, the chicken gut would not be the optimal site to expect the potential resuscitation of VBNC stages; the chance for resuscitation would be better under exquisite laboratory conditions. That is what a recent study (4) has shown by reported resuscitation of VBNC *Campylobacter* cells in eggs. VBNC stages seem to possess potential importance if judged by such results; nevertheless, our findings devalue the role of VBNC stages under natural conditions.

We conclude that the ability of three saline-stored *C. jejuni* strains to multiply under in vitro laboratory conditions did not reflect the potential for colonization of chickens and that the infectivity weakened and terminated before transformation into a VBNC stage could occur. The results from this study point to skepticism about an important epidemiological role for injured or aged environmental *Campylobacter* cells, even those that can still be cultured, and to the negligible role of VBNC stages in *Campylobacter* epidemiology. The fact that the chicks for these experiments were obtained under the same conditions as chicks for stocking at Danish broiler farms makes the results of this study relevant to ordinary broiler production.

Even high levels of anti-*Campylobacter* OMP serum antibodies did not protect chicks from *Campylobacter* colonization when inoculated with doses of less than 100 CFU.

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REFERENCES

1. Achen, M., T. Y. Morishita, and E. C. Ley. 1998. Shedding and colonization of *Campylobacter jejuni* in broilers from day-of-hatch to slaughter age. *Avian Dis.* **42**:732-737.
2. Adak, G. K., J. M. Cowden, S. Nicholas, and H. S. Evans. 1995. The Public Health Laboratory Service national case-control study of primary indigenous sporadic cases of campylobacter infection. *Epidemiol. Infect.* **115**:15-22.
3. Buswell, C. M., Y. M. Herlihy, L. M. Lawrence, J. T. McGuigan, P. D. Marsh, C. W. Keevil, and S. A. Leach. 1998. Extended survival and persistence of *Campylobacter* spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and rRNA staining. *Appl. Environ. Microbiol.* **64**:733-741.
4. Cappelier, J. M., J. Minet, C. Magras, R. R. Colwell, and M. Federighi. 1999. Recovery in embryonated eggs of viable but nonculturable *Campylobacter jejuni* cells and maintenance of ability to adhere to HeLa cells after resuscitation. *Appl. Environ. Microbiol.* **65**:5154-5157.
5. Cawthraw, S. A., T. M. Wassenaar, R. Ayling, and D. G. Newell. 1996. Increased colonization potential of *Campylobacter jejuni* strain 81116 after passage through chickens and its implication on the rate of transmission within flocks. *Epidemiol. Infect.* **117**:213-215.
6. Eberhart-Phillips, J., N. Walker, N. Garrett, D. Bell, D. Sinclair, W. Rainger, and M. Bates. 1997. Campylobacteriosis in New Zealand: results of a case-control study. *J. Epidemiol. Community Health* **51**:686-691.
7. Engberg, J., P. Gerner-Smidt, F. Scheutz, E. M. Nielsen, S. L. W. On, and K. Mølbak. 1998. Water-borne *Campylobacter jejuni* infection in a Danish town—a 6-week continuous source outbreak. *Clin. Microbiol. Infect.* **4**:648-656.
8. Fearnley, C., R. D. Ayling, and D. G. Newell. 1994. The failure of viable but non-culturable *Campylobacter jejuni* to colonize the chick model. Report of a WHO consultation on epidemiology and control of campylobacteriosis in animals and humans, Bilthoven, 25-27 April 1994. 85-87. World Health Organization, Geneva, Switzerland.
9. Hald, B., A. Wedderkopp, and M. Madsen. 2000. Thermophilic *Campylobacter* spp. in Danish broiler production: a cross sectional survey and a retrospective analysis of risk factors for occurrence in broiler flocks. *Avian Pathol.* **29**:123-131.
10. Harrington, C. S., F. M. Thomson-Carter, and P. E. Carter. 1997. Evidence for recombination in the flagellin locus of *Campylobacter jejuni*: implications for the flagellin gene typing scheme. *J. Clin. Microbiol.* **35**:2386-2392.
11. Hobbie, J. E., R. J. Daley, and S. Jasper. 1976. Use of Nucleopore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* **33**:1225-1228.
12. Hopkins, R. S., R. Olmsted, and G. R. Istre. 1984. Endemic *Campylobacter jejuni* infection in Colorado: identified risk factors. *Am. J. Public Health* **74**:249-250.
13. Jones, D. M., E. M. Sutcliffe, and A. Curry. 1991. Recovery of viable but non-culturable *Campylobacter jejuni*. *J. Gen. Microbiol.* **137**:2477-2482.
14. Kapperud, G., E. Skjerve, N. H. Bean, S. M. Ostroff, and J. Lassen. 1992. Risk factors for sporadic *Campylobacter* infections: results of a case-control study in southeastern Norway. *J. Clin. Microbiol.* **30**:3117-3121.
15. Korhonen, L. K., and P. J. Martikainen. 1991. Comparison of the survival of *Campylobacter jejuni* and *Campylobacter coli* in culturable form in surface water. *Can. J. Microbiol.* **37**:530-533.
16. Lazaro, B., J. Carcamo, A. Audicana, I. Perales, and A. Fernandez-Astorga. 1999. Viability and DNA maintenance in nonculturable spiral *Campylobacter jejuni* cells after long-term exposure to low temperatures. *Appl. Environ. Microbiol.* **65**:4677-4681.
17. Medema, G. J., F. M. Schets, A. W. van de Giessen, and A. H. Havelaar. 1992. Lack of colonization of 1 day old chicks by viable, non-culturable *Campylobacter jejuni*. *J. Appl. Bacteriol.* **72**:512-516.
18. Nachamkin, I., K. Bohachick, and C. M. Patton. 1993. Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. *J. Clin. Microbiol.* **31**:1531-1536.
19. Newell, D. G., H. McBride, F. Saunders, Y. Dehele, and A. D. Pearson. 1985. The virulence of clinical and environmental isolates of *Campylobacter jejuni*. *J. Hyg. Camb.* **94**:45-54.
20. Nordentoft, S., H. Christensen, and H. C. Wegener. 1997. Evaluation of a fluorescence-labeled oligonucleotide probe targeting 23S rRNA for in situ detection of *Salmonella* serovars in paraffin-embedded tissue sections and

- their rapid identification in bacterial smears. *J. Clin. Microbiol.* **35**:2642–2648.
21. Palmer, S. R., P. R. Gully, J. M. White, A. D. Pearson, W. G. Suckling, D. M. Jones, J. C. Rawes, and J. L. Penner. 1983. Water-borne outbreak of campylobacter gastroenteritis. *Lancet* **i**:287–290.
  22. Pearson, A. D., M. Greenwood, T. D. Healing, D. Rollins, M. Shahamat, J. Donaldson, and R. R. Colwell. 1993. Colonization of broiler chickens by waterborne *Campylobacter jejuni*. *Appl. Environ. Microbiol.* **59**:987–996.
  23. Penner, J. L., J. N. Hennessy, and R. V. Congi. 1983. Serotyping of *Campylobacter jejuni* and *Campylobacter coli* on the basis of thermostable antigens. *Eur. J. Clin. Microbiol.* **2**:378–383.
  24. Rollins, D. M., and R. R. Colwell. 1986. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Appl. Environ. Microbiol.* **52**:531–538.
  25. Saha, S. K., S. Saha, and S. C. Sanyal. 1991. Recovery of injured *Campylobacter jejuni* cells after animal passage. *Appl. Environ. Microbiol.* **57**:3388–3389.
  26. Shanker, S., A. Lee, and T. C. Sorrell. 1988. Experimental colonization of broiler chicks with *Campylobacter jejuni*. *Epidemiol. Infect.* **100**:27–34.
  27. Stern, N. J., J. S. Bailey, L. C. Blankenship, N. A. Cox, and F. McHan. 1988. Colonization characteristics of *Campylobacter jejuni* in chick coeca. *Avian Dis.* **32**:330–334.
  28. Stern, N. J., D. M. Jones, I. Wesley, and D. M. Rollins. 1994. Colonization of chicks by non-culturable *Campylobacter* spp. *Lett. Appl. Microbiol.* **13**:333–336.
  29. Tagawa, Y., M. Haritani, H. Ishikawa, and N. Yuasa. 1993. Characterization of a heat-modifiable outer membrane protein of *Haemophilus somnus*. *Infect. Immun.* **61**:1750–1755.
  30. Terzieva, S. I., and G. A. McFeters. 1991. Survival and injury of *Escherichia coli*, *Campylobacter jejuni*, and *Yersinia enterocolitica* in stream water. *Can. J. Microbiol.* **37**:785–790.
  31. van de Giessen, A. W., C. J. Heuvelman, T. Abee, and W. C. Hazeleger. 1996. Experimental studies on the infectivity of non-culturable forms of *Campylobacter* spp. in chicks and mice. *Epidemiol. Infect.* **117**:463–470.
  32. Young, C., R. Ziprin, M. Hume, and L. Stanker. 1999. Dose response and organ invasion of day-of-hatch leghorn chicks by different isolates of *Campylobacter jejuni*. *Avian Dis.* **43**:763–767.