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Research Article

Colonization of Day-Old Broiler Chicks with *Campylobacter coli* through Different Inoculation Routes

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Abstract

Newly hatched broiler chicks may be exposed to *Campylobacter* from various sources in the hatchery and grow-out environments. Chicks may come in contact with *Campylobacter* in the air or in the droppings of other birds which chicks may eat or sit on. It is not clear how airborne, cloacal and oral exposure to *Campylobacter* may affect subsequent cecal colonization. In this study, a marker strain of *Campylobacter coli*, naturally gentamicin resistant (Cc^{GR}), was introduced into 585 day-of-hatch chicks through one of four body openings {mouth (with and without $CaCO_3$), nasal passage, eye and cloaca} to simulate oral, cloacal and airborne exposure. *Campylobacter coli*^{GR} was introduced by each route of exposure at three different inoculum levels (approximately 2 x 10^1, 2 x 10^2 and 2 x 10^3 colony forming units (cfu)/bird). All chicks were humanely euthanized 7 d, ceca were removed and sampled for the presence and numbers of Cc^{GR} by serial dilution onto Campy-cefex agar plates with 200 ppm gentamicin. Three replications were conducted (n=225, n=225 and n=135 for experiments 1, 2 and 3 respectively). All routes of exposure that were tested resulted in cecal colonization of 7 d old broilers. The nasal passage produced the lowest level of cecal colonization requiring higher inoculum levels of Cc^{GR} for colonization. These data suggest that Cc^{GR} can readily colonize the ceca of day-of-hatch broiler chicks when exposed by mouth, cloaca, or breathing/blinking in airborne cells. Therefore, multiple intervention strategies may be required to interrupt exposure and colonization of young broilers by *Campylobacter*.

ABBREVIATIONS

mL: Milliliter; cfu: Colony Forming Unit; Cc^{GR}: Gentamicin Resisitant *Campylobacter coli*; h: Hours; d: Days; CCAG: *Campylobacter* Cefex Agar W/ Gentamicin; NB: Nutrient Broth; TB: Tecra® Broth; IU: Isolation Unit; w/v: Weight/Volume; C: Celsius

INTRODUCTION

Campylobacter is a leading cause of bacterial induced diarrheal disease in the United States and worldwide [1,2]. *Campylobacter jejuni* is the leading organism isolated from humans and *C. coli* is the second leading causative agent for human diarrheal illness [3]. Poultry has long been implicated as a major source of transmission for *Campylobacter* to humans [4].

The routes of entry of this organism to broiler chickens have not been studied but Cox et al., [5] did examine the ability of *Salmonella* species to enter and colonize the ceca of broiler chicks by various body openings. They found that marker strains of nalidixic acid resistant *Salmonella* were able to enter and colonize ceca of day-of-hatch broiler chicks through the mouth, eyes, nares, and cloaca, consistently. Bailey et al. [6], found that after intracloacal and oral inoculation, *Salmonella* could be recovered from the thymus, spleen, liver/gall bladder, ceca and bursa in as little as 1 h post inoculation. Cox et al. [7], found naturally occurring *Salmonella* and *Campylobacter* in the internal organs of commercial broilers aged 6 and 8-week.

Research efforts to diminish the presence of this organism on processed poultry have had limited success partly because there is limited information regarding the entry of *Campylobacter* into commercial poultry flocks, particularly because it is difficult to detect naturally occurring *Campylobacter* until 2-3 weeks of age [8]. Husbandry, cleaning and disinfection of grow-out houses, ventilation, and colonization of parent flocks are factors often examined when trying to eliminate or delay the colonization of chicks in a broiler house [9, 10, 11, 12]. The goal would be to delay the event sufficiently to avoid any contaminated birds entering the processing plant.

The purpose of the experiment was to determine likely avenues of entry of *Campylobacter* into day of hatch broiler chicks.

MATERIALS AND METHODS

Bacterial strains, growth and maintenance

Maintenance: Gentamicin resistant *Campylobacter coli*^{GR} (Cc^{GR}) [13] was maintained on Campy-CEFEX agar with 200 µg/mL gentamicin (CCAG) added. Cc^{GR} were grown at 42°C for

48 h in a microaerobic atmosphere (85% N, 10% O_2, 5% CO_2). Cc^{GR} were maintained frozen at -80°C in nutrient broth (NB, Becton-Dickinson, Franklin Lakes, NJ) with 15% glycerol (Sigma Chemical Co., St. Louis, MO).

Inoculum Preparation: Inocula were prepared according to laboratory standard operating procedures. Briefly, a culture was streaked from the stock culture maintained in the -80°C freezer onto CCAG, incubated at 42°C for 48 h under microaerobic conditions. Inocula were prepared from this plate by choosing several well isolated colonies and suspending these colonies in enough sterile 0.85% saline (NaCl, Sigma Chemical Co., St. Louis, MO) to create a suspension with an optical density of 0.20 at 540_{nm} with a Spect-20 (Milton-Roy Spectrophotometer, Thermo Spectronics, Madison, WI). This provides approximately 2.0 x 10^8 colony forming units (cfu)/mL of solution as determined by use of a standard curve (data not shown). Serial dilutions were prepared to approximately one log cfu/mL above the desired inoculum levels of 10^1, 10^2 and 10^3 cfu/bird; 0.1 mL of this solution was used to inoculate the chicks via various routes. Chicks (n=15 per isolation unit (IU)) were inoculated with 0.1 mL orally (with and without 5% $CaCO_3$ buffer (Sigma Chemical Co.)), ocularly (through the eye, drop wise with the inoculum allowed to enter before another drop was administered), intranasally (through the nares, drop wise with the inoculum allowed to enter before another drop was administered) or intracloacally (by insertion of approximately 1 cm of a tuberculin syringe into the vent of the chick). The oral inoculation was administered with and without 5% $CaCO_3$. The addition of 5% $CaCO_3$ was to mimic the effect of eggshell as a buffer when ingested with bacteria in a hatchery environment. The inocula were plated after appropriate serial dilutions onto CCAG for enumeration and incubated at 42°C for 48 h under microaerobic conditions.

Animals and animal husbandry

All day of hatch chicks were obtained from a local commercial broiler hatchery and transported on clean cardboard bedding pads in reusable chick transportation trays. Chick transport bedding pads (n=3) were sampled on day of placement by enrichment in 500 mL of Tecra Broth™ (TB, 3M Corporation, St. Paul, MN) and subsequent plating on Campy-CEFEX agar plates without gentamicin for detection of naturally occurring *Campylobacter*. Ceca from ten (n=10) chicks were aseptically removed and sampled as described below. Chicks were housed in separate IU on wire mesh flooring (15 chicks per IU) with *ad libitum* access to feed (non-medicated starter/grower crumbles and pellets, University of Georgia Poultry Science Feed Mill, Athens, GA) and water on a 24 h light regimen. Standard husbandry practices for growth were followed with birds being culled for disease and physical abnormalities which would lead to poor growth performance. At 7 d, all birds were humanely euthanized by cervical dislocation.

Isolation and enumeration

Seven (7) days post inoculation, all chicks per IU were humanely euthanized by cervical dislocation, ten chicks (n=10) were surface disinfected with 70% ethanol (Pharmco-Aaper®, Shelbyville, KY) and the ceca aseptically removed. Ceca were placed into Stomacher 80® sample bags (Seward Laboratory Systems, Inc., Port Saint Lucie, FL) and transported to the laboratory on ice. In the laboratory, ceca were weighed individually, macerated, diluted 1:10 (w/v) with TB, stomached for 60 s and serially diluted in 0.85% sterile saline and plated in duplicate on CCAG for enumeration. All plates were incubated in a microaerobic environment at 42°C for 48 h. Bags containing ceca and TB were likewise incubated in a microaerobic environment at 42°C for 48 h as an enrichment. Enriched cecal samples were streaked for isolation onto CCAG to detect low levels of *Campylobacter* spp. One of each colony type per plate was examined by wet mount microscopy and latex agglutination (Microgen *Campylobacter* Latex Agglutination Kit, Microbiology International, Frederick, MD) for confirmation as *Campylobacter* spp.

Experimental design

Three replications were conducted. In replications 1 and 2 three IU (n=15 birds) were used for each of three inoculum levels (10^1, 10^2 and 10^3 cfu/bird) and each of four body openings (n=225/replication). For each replication four body openings (mouth, eye, nares and cloaca) were used as the routes of of entry for the inoculum. Ten chicks (n=10) per treatment/IU were sampled for the presence of Cc^{GR} by serial dilution and enrichment to determine presence or absence of Cc^{GR} in the ceca. Average cfu/mL of cecal material was calculated by counting the appropriate dilution. Samples with no growth on enumeration plates were streaked for isolation from the enriched samples onto fresh CCAG plates and incubated at 42°C for 48 h, microaerobically. Any sample with no visible growth after incubation of the enriched sample was determined to be negative and assigned an average colonization value of 0. Based on the data showing 100% colonization with the intracloacal route of inoculation in Replications 1 and 2, Replication 3 used only two IU (n=15 birds) and two inoculum levels, 10^1 and 10^3 cfu/bird for the oral, ocular and nasal routes of entry and only one IU (n=15 birds) for the 10^3 cfu/bird for the intracloacal route. This reduction of animals used is in line with the USDA, ARS guidelines for the humane use of animals.

RESULTS AND DISCUSSION

Cox et al. [13], identified a marker strain of Cc^{GR} which was found to have a stable resistance to gentamycin and to be a reliable colonizer of day of hatch broiler chicks and this strain was used in this experiment. Results of inoculation by oral gavage with and without $CaCO_3$ are shown in Table 1. When day old broiler chicks were orally gavaged with 10^1 cfu/bird, only birds in one of the three groups were colonized. Birds in the subsequent groups which received 10^2 or 10^3 cfu/bird were colonized and with an average of log 7.2 cfu/mL of Cc^{GR} marker in the ceca. When $CaCO_3$ was included in the inoculation broth, all birds challenged with Cc^{GR} were colonized by the marker strain with an average of $\log_{10}$ 7.7 cfu/mL in the cecal material. Calcium carbonate appears to serve as a buffering agent against the hostile pH of the gizzard which Cox et al. [14], determined to be approximately pH 1.9

Table 1: Cecal colonization of day-old broiler chicks with CC^{GR} following oral gavage with and without 5% $CaCO_3$.

Replication	$CaCO_3$	Inoculation Level	# Positive/ # Tested[a]	Average Cecal Colonization[b]
1	None	1.5×10^1	0/10	0
		1.5×10^2	10/10	8.3
		2.0×10^3	10/10	7.0
2	None	1.6×10^1	10/10	7.6
		1.8×10^2	10/10	6.8
		2.0×10^3	10/10	6.9
3	None	1.8×10^1	0/10	0
		2.0×10^3	10/10	7.1
1	5%	1.5×10^1	10/10	8.1
		1.5×10^2	10/10	8.2
		2.0×10^3	10/10	6.9
2	5%	1.6×10^1	10/10	8.0
		1.8×10^2	10/10	7.9
		2.0×10^3	10/10	6.9
3	5%	1.8×10^1	10/10	8.3
		2.0×10^3	10/10	6.9

[a] Number of ceca positive for *C. coli*/Number of ceca and cecal content tested.
[b] Log_{10} cfu of *C. coli* per gram of ceca and cecal content.

Table 2: Cecal colonization of day-old broiler with CC^{GR} following inoculation of the eye.

Replication	Inoculation Level	# Positive / # Tested[a]	Average Cecal Colonization[b]
1	1.5×10^1	0/10	0
	1.5×10^2	10/10	7.4
	2.0×10^3	10/10	7.0
2	1.6×10^1	10/10	8.0
	1.8×10^2	10/10	6.8
	$2.0\ 10^3$	10/10	7.0
3	1.8×10^1	10/10	7.0
	2.0×10^3	10/10	7.0

[a] Number of ceca positive for *C. coli*/Number of ceca and cecal content tested.
[b] Log_{10} cfu of *C. coli* per gram of ceca and cecal content.

Table 3: Cecal colonization of day-old broiler with CC^{GR} following inoculation of the nares.

Replication	Inoculation Level	# Positive / # Tested[a]	Average Cecal Colonization[b]
1	1.5×10^1	0/10	0
	1.5×10^2	10/10	7.7
	2.0×10^3	10/10	7.0
2	1.6×10^1	0/10	0
	1.8×10^2	3/10	3.1
	2.0×10^3	10/10	6.9
3	1.8×10^1	0/10	0
	2.0×10^3	10/10	6.7

[a] Number of ceca positive for *C. coli*/Number of ceca and cecal content tested.
[b] Log_{10} cfu of *C. coli* per gram of ceca and cecal content.

Table 4: Cecal colonization of day-old broiler with CC^{GR} following intra-cloacal inoculation.

Replication	Inoculation Level	# Positive / # Tested[a]	Average Cecal Colonization[b]
1	1.5×10^1	10/10	6.9
	1.5×10^2	10/10	6.9
	2.0×10^3	10/10	6.9
2	1.6×10^1	10/10	8.1
	1.8×10^2	10/10	6.9
		d	
3	2.0×10^3	10/10	6.9

[a] Number of ceca positive for *C. coli*/Number of ceca and cecal content tested.
[b] Log_{10} cfu of *C. coli* per gram of ceca and cecal content.

by direct measurements. These results are somewhat different than those observed with a *Salmonella* marker when $CaCO_3$ was added to the inoculum [5]. Calcium carbonate did not increase the chances of a chick becoming colonized with *Salmonella* but it appears to increase the chance a chick may be colonized with *Campylobacter*. This is understandable since *Campylobacter* is a more fragile microorganism and may be more sensitive to the acidic conditions in the provetnriculus and/or gizzard.

Intraocular inoculation of Cc^{GR} ranged from 15 to 2000 cfu/bird and resulted in the colonization of 70 of the 80 chicks challenged through this route (Table 2). Additionally, this suggests it is possible to produce potential seeder birds by entry of low numbers of bacteria such as *Campylobacter* and *Salmonella* [5] into the eyes of young chicks. This route of entry is significant in that there may be many airborne bacteria present in the various environments that young chicks are exposed to, such as hatching cabinets, hatcheries and grow-out houses. This provides an ideal environment for early colonization of birds without an oral challenge.

Table 3 shows the cecal colonization of day of hatch chicks when Cc^{GR} were introduced into the trachea via the nares. This route of inoculation produced fewer colonized chicks with none colonized at the lowest inoculation level of approximately 10^1 cfu/bird (statistical difference was not determined). Similar to intraocular inoculation this route of entry may be of biological significance because of airborne microorganisms in the chick's environment and the nares provide a moist body opening for bacteria to enter the chick's body. Cox et al. [5], used two different methods for inoculating the respiratory tract of chicks, using a small tube through the cleft palette and by fogging with a fine mist, and the results with *Salmonella* obtained were similar to the data in this experiment where the lower inoculum levels did not consistently result in colonization.

When Cc^{GR} was introduced intracloacally, all of the chicks were colonized even when the inocula contained only 15 cfu/bird (Table 4). This route of entry is significant because of the anti-peristalsis present in the cloaca of the young chicks [15]. Microorganisms on any surface could be introduced by this anti-peristaltic action into the cloaca and not be subject to the low pH, high acidity, of the proventriculus and/or gizzard of the chick's intestinal tract.

Regardless of the route or source, preventing the exposure of newly hatched chicks to *Campylobacter* is critical because the young bird lacks a mature gut microflora and as such is highly susceptible to colonization. This study clearly demonstrates that a marker strain of *Campylobacter* (Cc^{GR}) can colonize the intestinal tract of young broiler chicks through an assortment of body openings. There is potential for seeder birds to be produced by bacterial contamination which may occur through any of the four body openings utilized in this study and may occur early in the chick's life. This contamination can then be spread throughout the broiler house to both the outside and/or inside of the flock mates present. Further studies are needed to develop intervention strategies to prevent colonization by any and/or all of these openings of the body.

CONCLUSION

Gentamycin resistant *Campylobacter coli* can readily colonize the ceca of day-of-hatch chicks by different routes of inoculation and in particular, routes other than the fecal – oral route.

As few as 10^1 cfu/ bird are able to colonize the ceca of day-of-hatch chicks through three of the four body openings tested.

Calcium carbonate appears to act as a buffer to the low pH of the gastric system allowing Cc^{GR} to more easily colonize the ceca.

Preventing *Campylobacter* species from colonizing chicks and chickens is going to take more research in laboratories, in the hatcheries, on farms and in the processing plant. Having this gentamycin resistant marker will make this research easier.

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