Comparison of Challenge Models for Determining the Colonization Dose of Campylobacter jejuni in Broiler Chicks

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ABSTRACT Coprophagous activity is normal among broiler chickens. The purpose of this study was to compare an individually housed chick model (where bird-to-bird coprophagia was prevented) to a group-housed chick model (where bird-to-bird coprophagia was allowed) for determining estimates of the number of Campylobacter jejuni RM1221 necessary to colonize 50% of broiler chicks inoculated (colonization dose 50% or CD50).

Campylobacter jejuni RM1221 was orally administered in measured doses to newly hatched chicks. The chicks were housed either individually in cages designed to minimize coprophagous activity or in isolation units containing groups of birds where coprophagia was allowed. The birds were killed and analyzed for Campylobacter in the ceca on d 7 postinoculation. The CD50 was calculated, and results from the 2 models were compared. Elimination of transmission of Campylobacter, through coprophagia or other means, led to a more clear determination of the estimated CD50 of about 524 cfu of C. jejuni RM1221 as demonstrated in the individually housed chick model. Bayesian inference based on the beta-Poisson statistical modeling procedures were found to be superior to standard single-hit dose-response modeling for estimation of the CD50. This study demonstrated that the individual bird challenge model is superior to the group challenge model for trials designed to determine colonization dose.

Key words: broiler, Campylobacter jejuni, individually housed chick model, group-housed chick model

INTRODUCTION

Coprophagia (the oral ingestion of fecal matter) is a normal activity of broiler chickens, and the spread of zoonotic pathogens (poultry and human) through the fecal oral route is well documented. Hyun and Sakaguchi (1989) implicated coprophagous activity in cases of chicken botulism. Folz et al. (1986) suggested reducing coccidiosis in chickens by giving an antico prophagia drug to the birds to prevent the ingestion of infective oocysts. An increased Salmonella spp. incidence in the crop of birds has been reported after feed withdrawal and is likely due to increased coprophagous activity (Barnhart et al., 1999; Corrier et al., 1999). Coprophagia has also been implicated in the spread of Campylobacter jejuni through commercial chickens (Montrose et al., 1985; Line, 2006).

Campylobacter jejuni is a gram-negative human foodborne pathogen of primary importance (Altekruse et al., 1999). Poultry are frequently contaminated with C. jejuni during production with the majority of commercial US flocks positive for the organism by the time the birds reach market age at about 6 wk (Stern et al., 2001). There are currently few effective on-farm interventions for reducing colonization of poultry with C. jejuni, and there is a need for effective interventions that may be practically applied in the poultry industry to reduce colonization of poultry with C. jejuni and subsequently reduce consumer exposure to this pathogen. Campylobacter jejuni is a commensal organism in poultry, and, as such, colonization causes no apparent health problems for the bird (Beery et al., 1988). The organism has a low infective dose (Ruiz-Palacios et al., 1981; Stern et al., 1988; Wallis, 1994; Young et al., 1999); therefore, administration of small populations of C. jejuni results in rapid cecal colonization of the bird followed by fecal shedding of very large populations of the microorganism frequently approaching 10⁷ cfu/g of feces (Achen et al., 1998; Dhillon et al., 2006). This increase in the overall population of Campylobacter spp. present in the environment is important, and the ingestion of contaminated feces through coprophagous activity results in rapid spread of the pathogen among members of the flock (Shanker et al., 1990; Van Gerwe et al., 2005; Line, 2006).
The rapid spread of *Campylobacter* spp. through groups of birds housed together can have an effect on scientific studies designed to determine the minimum colonization dose, or the effectiveness of a proposed treatment for reducing *Campylobacter* spp. colonization. Several investigations have been conducted in an effort to reduce populations of foodborne pathogens in poultry on the farm so that fewer numbers of pathogens enter the processing facility and subsequent consumer exposure is diminished (Stern, 1994; Corrier et al., 1995; Morishita et al., 1997; Line et al., 1998; Mead, 2000). The results of studies investigating the effect of on-farm intervention technologies can be confounded by coprophagic activity, if such activity results in the reexposure of birds from which the pathogen was previously eliminated. Likewise, studies to determine the minimum colonization dose of a pathogen or investigations of the population of a specific pathogen necessary to colonize 50% of experimental animals inoculated (colonization dose 50%, CD50) can be misled by subsequent coprophagic activity among animals. The purpose of this study was to compare an individually housed chick model (where bird-to-bird coprophagia was prevented) to a group-housed chick model (where bird-to-bird coprophagia was allowed) for determining estimates of the CD50 for *C. jejuni* RM1221 in broiler chicks.

**MATERIALS AND METHODS**

**Campylobacter Strain**

*Campylobacter jejuni* RM1221 was obtained from Rob Mandrell (Fouts et al., 2005) and was stored frozen at −80°C in an 80% glycerol solution. The culture was prepared for the chick challenge experiments by rapidly thawing an aliquot of the frozen *C. jejuni* solution, streaking it for isolation on Brucella agar containing ferrous sulfate, sodium metabisulfite and sodium pyruvate, and incubating for 48 h at 42°C under microaerobic conditions (5% O2, 10% CO2, and 85% N2). Cells were harvested and diluted to an approximate density of 10^8 cfu/mL using a spectrophotometer and standard curve. Serial dilutions were made in cold (4°C) PBS to achieve the target challenge populations. The inocula were kept on ice <1 h before oral gavage of chicks.

**Experimental Design and Challenge Trials**

Commercial broiler chicks (90, Ross × Ross) were obtained from a local hatchery on the day of hatch and transported (<1 h) to the Agricultural Research Service animal facility in Athens, Georgia. Chicks were randomly divided into 3 treatment groups of 30 birds each. One group of chicks was administered a high challenge level of about 10^4 cfu of *C. jejuni* RM1221 per bird in 0.1 mL by oral gavage. A second group similarly received a lower challenge dose of about 10^2 cfu/bird. The third group served as negative controls and received 0.1 mL of sterile PBS. Each treatment group was then further subdivided for placement of single chicks into individual cages (10 total cages per treatment) where no bird-to-bird coprophagia was possible, or in duplicate isolation units containing 10 birds each where coprophagic activity was allowed to occur.

The 10 individually housed birds were maintained in modified wire-floored rat cages (about 30 × 30 × 40 cm) equipped with bell jars (500 mL) for water and feed pans (12-cm diameter). The individual cages (30) were held in a metal rack and placed in a microbiologically isolated environmental control chamber. Temperature was maintained at approximately 45°C by controlling the incoming air temperature via a digital thermostat and electric heater strip in the incoming airflow. The air entering the positive-pressure units was high efficiency particulate air-filtered (Abatement Technologies, Suwanee, GA) as it was forced into the chamber and also filtered as it exited to maintain microbiological isolation. A disinfectant foot bath was maintained at the door to the chamber, and animal caretakers and technical personnel were required to wear protective coveralls, boots, and gloves when entering the chamber and to change gloves between cages to avoid cross contamination.

The chicks used in group challenges were housed in isolation units (about 1 m^2) equipped with high efficiency particulate air-filtered air supplies and commercial-type nipple drinkers (Ziggity Systems Inc., Middlebury, IN). These units had solid floors to facilitate coprophagic activity. The temperature was controlled at about 45°C using commercial brooder lamps. Proper procedures were followed to maintain microbiological isolation for these groups of birds also including changing of gloves between units to avoid cross contamination. All chicks were given access to water and standard nonmedicated broiler starter feed ad libitum. Additionally, *Campylobacter* spp. colonization was monitored by humanely euthanizing the chicks by cervical dislocation on d 7 postinoculation and aseptically removing the ceca for microbiological analysis.

**Microbiological Analysis**

The presence of *Campylobacter* spp. in the ceca was determined by diluting the ceca 1:3 (wt/vol) in PBS (pH 7.2) and homogenizing in a Stomacher 80 laboratory blender (Seward Ltd., London, UK) followed by direct-plating of 10-fold serially diluted samples on Campy-Line agar (Line, 2001). Plates were incubated at 42°C for 36 to 48 h under microaerobic conditions (5% O2, 10% CO2, 85% N2). After incubation, typical *Campylobacter* spp. colonies were enumerated and confirmed by microscopic wet mount and latex agglutination using a Microscreen *Campylobacter* Agglutination Kit (Microgen Bioproducts, Camberley, Surrey, UK) for confirmation as necessary. The minimum detection limit was 30 cfu per gram of cecal contents.
This experiment was repeated in its entirety a total of 4 times. All trials were approved by the Institutional Animal Care and Use Committee (PMSRU-01–06).

**flaA Short Variable Region DNA Sequence Analysis**

In an effort to ensure that the *C. jejuni* isolate, RM1221, used to inoculate the birds was the same as the isolates excreted from the birds, *flaA* short variable region (SVR) subtype analysis was performed (Meinersmann et al., 1997). Approximately 20 *Campylobacter* spp. isolates, collected during each sampling trial, were randomly chosen from plates. Isolated colonies of *Campylobacter* spp. were suspended in 300 µL of sterile H2O and boiled for 10 min. Ten microliters of each boiled cell suspension was used as template for *flaA* SVR PCR with the following primers: FLA-242F, 5’CTAGTGATGACAAATTWAAATG5’ and FLA625RU, 5’CAAGWCCGCTTCWCACTGAAAG5’ (Meinersmann et al., 1997). A 35-cycle reaction was used with 1 min of denaturing at 96°C, 1 min of annealing at 52°C, and a 1 min of extension at 72°C. The resulting product was approximately 425 bp. Sequence data were generated using either the FLA242F primer or the FLA625RU primer with the Big-Dye Dye-Terminator Cycle Sequencing Kit (ABI-PE, Foster City, CA). Data were assembled with Sequencher 4.7 (GeneCodes Corp., Ann Arbor, MI) and aligned using ClustalX (Thompson et al., 1994). Aligned sequences were compared and dendrograms generated using the neighbor-joining algorithm with HKY85 distance measurements in PAUP 4.0b, where PAUP = phylogenetic analysis using parsimony (Swofford, 1988).

**Pulsed Field Gel Electrophoresis Analyses**

To further ensure that the *C. jejuni* isolate, RM1221, used to inoculate the birds was the same as the isolates excreted from the birds, pulse field gel electrophoresis was performed as described previously (Ribot et al., 2001). Resulting banding patterns were analyzed using neighbor-joining analysis with 1% band position tolerance in the MultiAnalyst2 program (BioRad, Hercules, CA).

**Statistical Analysis**

Arithmetic estimates of the numbers of *Campylobacter* bacteria were transformed into logarithmic format. Statistical means and standard deviations were determined using SigmaStat statistical software (Jandel Scientific, San Rafael, CA). Estimates of the CD50 for *C. jejuni* RM1221 were obtained through Bayesian inference based on the standard single-hit and beta-Poisson dose-response models (French et al., 2002). Both models are based on the hypothesis of independent action: the probability of colonization is dependent only on the dose and the (small) probability of an individual bacterium successfully colonizing the host. Under the single-hit model, this individual bacterium colonization probability is constant. Heterogeneity between different hosts can lead to variability in the individual bacterium colonization probability between hosts. The beta-Poisson model, strictly an approximation to the hypergeometric dose-response model (Teunis and Havelaar, 2000), allows for host heterogeneity by modeling the individual bacterium colonization probability as a beta distribution. A constant prior distribution was used, to place full weight on the data and make the estimation procedure exactly equivalent to a maximum likelihood method (Gelman et al., 1995). Estimates were obtained separately for the individually housed chicks, both replicates of co-housed birds, and for the pooled data from these 2 replicates. Strictly, these dose-response models should not be applied to data from co-housed birds, because they do not account for the possibility of transmission. Nevertheless, here we apply the dose-response models naively to co-housed dose-response data to explore the effect of transmission on the apparent dose-response relationship. The fitted posterior distribution (the likelihood of observing the given data under a specified model) was used for comparison of the uncertainty in the estimates of CD50 and to calculate 95% credibility intervals. All statistical analyses were carried out using the GNU R package (R Development Core Team, 2007).

**RESULTS**

*Campylobacter* isolates recovered from birds in all trials were determined to be the same *flaA* SVR genotype and pulsed field gel electrophoresis genotype as the original isolate, RM1221, used for inoculation. All negative control birds remained negative for *Campylobacter* spp. throughout the trials in both the individual and group bird experiments.

Colonization results from birds housed individually and therefore not allowed any bird-to-bird coprophagic activity demonstrated a simple direct dose-response relationship between increasing challenge dose and probability of in vivo colonization. The dose-response relationship for *C. jejuni* RM1221 was explored by fitting the single-hit and beta-Poisson dose-response models. Under the single-hit model, the CD50 was estimated to be log10 3.47 (95% credibility interval of 3.3 to 3.63). The small credibility interval associated with the single-hit model belies a poor fit to the apparent slope of the dose-response function. The maximum likelihood fit of the beta-Poisson model, with the extra flexibility afforded by a second parameter, captures the shape of the data more convincingly and gives a smaller CD50 estimate of log10 2.67 (95% credibility interval of 2.47 to 2.89). These differing estimates can be resolved by treating the greatest dose group of chicks as an outlier (Figure 1). Removing this single group leads to a more convincing dose-response relationship and estimated CD50 of...
log\(_{10}\) 2.76 (95% credibility interval of 2.61 to 2.94). In contrast, removal of this outlier has negligible effect on estimates of CD\(_{50}\) under the beta-Poisson model, which is then adjusted to log\(_{10}\) 2.70 (95% credibility interval of 2.42 to 2.95).

By contrast, results from challenge dose experiments conducted with groups of birds housed together were more consistent with the dose-response relationship predicted by the single-hit model. Groups of chicks demonstrate a pronounced all-or-nothing response (Figure 2) more typical of previously published dose-response studies (Cawthraw et al., 1996) and over a narrower range of colony-forming units than for the individually housed chicks (Figure 1). Estimates of CD\(_{50}\) derived from the data pooled across both replicates were consistent between the single-hit and beta-Poisson models of log\(_{10}\) 2.52 (95% credibility interval of 2.42 to 2.62) and log\(_{10}\) 2.35 (95% credibility interval of 2.18 to 2.50), respectively.

However, there was considerable variability between the 2 replicate experiments. Challenge doses delivered on the day of hatch ranging from approximately 130 cfu/chick to about 690 cfu/chick sometimes colonized 100% of the birds in the group and sometimes did not colonize any of the birds in the group upon analysis on d 7 (Table 1). The all-or-nothing response leads to distinct estimates of CD\(_{50}\) and a shifted dose-response relationship when the 2 replicates are treated independently (Figure 2). Under the single-hit model, replicate 1 provides a CD\(_{50}\) estimate of log\(_{10}\) 2.03 (95% credibility interval of 1.78 to 2.25), significantly lower than the estimate from the individually housed chicks and pooled group data. In contrast, replicate 2 provides a CD\(_{50}\) estimate of log\(_{10}\) 2.74 (95% credibility interval of

![Figure 1. Dose-response relationships for cecal colonization of individually housed chicks (n = 10) on d 7 after oral challenge with Campylobacter jejuni RM1221 on day of hatch. Shaded areas represent 95% credibility intervals based on 10,000 samples from the posterior distribution.](https://academic.oup.com/ps/article-abstract/87/9/1700/1548454/1703)

![Figure 2. Dose-response relationships for cecal colonization of co-housed chicks (n = 10) on d 7 after oral challenge with Campylobacter jejuni RM1221 on day of hatch. Shaded areas represent 95% credibility intervals based on 10,000 samples from the posterior distribution.](https://academic.oup.com/ps/article-abstract/87/9/1700/1548454)
2.66 to 2.82), significantly greater than the estimate from the individually housed chicks and pooled group data under the same model. The same pattern is seen under the beta-Poisson model, albeit with overlapping credibility intervals: for replicate 1, a CD50 estimate of log10 2.03 (95% credibility interval of 1.78 to 2.25) and replicate 2, a CD50 estimate of log10 2.52 (95% credibility interval of 2.28 to 2.76).

The initial challenge dose had no effect on the population of C. jejuni recovered from the ceca of colonized birds on d 7. The overall mean C. jejuni population in the colonized birds was log10 7.43 ± 0.32.

**DISCUSSION**

Coprophagous activity among groups of birds housed together can rapidly spread C. jejuni through a flock. Investigators have utilized colonized seeder birds to intentionally spread Campylobacter through experimental flocks (Shanker et al., 1990; Stern et al., 2001). Van Gerwe et al. (2005) investigated horizontal transmission through broiler flocks by placing Campylobacter-inoculated chicks with unoinoculated contact chicks and monitoring fecal samples for Campylobacter spp. The transmission rate was determined to be 1.04 new cases per colonized chick per day. This would imply that in a typical flock of 20,000 birds, the prevalence of Campylobacter spp. would increase from 5 to 95% within 6 d after Campylobacter spp. introduction. Indeed, contaminated litter alone can serve as a significant contamination source for Campylobacter spp. in broilers (Montrose et al., 1985). Line (2006) described the effects of relative humidity on the horizontal spread of Campylobacter spp. through groups of broiler chickens. Campylobacter jejuni-contaminated litter was used as the only source of inoculum and resulted in the rapid colonization of broiler chicks placed on the contaminated litter.

Transmission of Campylobacter spp., by coprophagous activity or other means, among birds housed in groups likely led to disparate results in the present challenge dose experiments and the distinctive all-or-nothing dose response. Comparing the results from the individually housed chick model (where bird-to-bird coprophagia was prevented) to the group challenge model (where bird-to-bird coprophagia was allowed) may offer a possible explanation for the conflicting results of the group challenge model. For example, when 2 groups of 10 birds each were challenged with about 180 cfu of C. jejuni/bird and housed together, all the birds in both groups were colonized by d 7. When 10 individually housed birds were challenged with the same level of inoculum of 180 cfu of C. jejuni/bird, only 30% of the birds were colonized by d 7. It is therefore reasonable to assume that only a few birds in the challenged group model were actually colonized by the original inoculum administered to the chicks. Campylobacter spp. populations increased in the gut of the colonized birds, which then defecated high populations of C. jejuni in their feces (often >107 cfu/g). This contaminated fecal material was then available to serve as a high-level inoculum source (several logs greater than the original inoculum) for other birds in the group perhaps not colonized by the original challenge delivered on the day of hatch by oral gavage. Rapid increases in the populations of Campylobacter spp. in the chicken intestinal tract have been observed, and increases of 2 to 5 log units in birds challenged with as few as 100 cfu have been reported (Stern et al., 1988; Dhillon et al., 2006).

The minimum colonization dose for Campylobacter spp. is thought to be low and may be affected by several factors including the age and strain of the bird, the strain of Campylobacter spp., and the physiological status of the microorganism. Estimates of the minimum colonization dose for C. jejuni in poultry range from as low as 35 cfu to about 10,000 cfu (Ruiz-Palacios et al., 1981; Stern et al., 1988; Wallis, 1994; Young et al., 1999). The maximum likelihood CD50 estimates we determined in the present study, for both housing methods (about 500 cfu), are within this range. How-

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**Table 1. Day 7 cecal colonization of chicks housed together in groups of 10 after oral challenge with Campylobacter jejuni RM1221 on day of hatch**

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<tr>
<th>Trial number</th>
<th>Estimated log10 cfu of C. jejuni RM1221 oral challenge dose on day of hatch</th>
<th>Chicks (%) cecally colonized with C. jejuni on d 7</th>
<th>Mean log10 cfu of C. jejuni g⁻¹ of cecal content on d 7</th>
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1ND = none detected. The limit of detection was ~30 cfu.
ever, the CD$_{50}$ estimate obtained from the individually housed experimental model required the killing of only 76 birds compared with the 160 birds required to achieve the same estimate from the co-housed model.

The shape of the dose-response relationship curve for C. jejuni RM1221 was modified when transmission between co-housed birds was allowed to occur. Compared with the individually housed data (Figure 1), the co-housed dose-response was more consistent with the single-hit dose-response model (Figure 2), with a steeper dose-response curve and a narrower width. Co-housing appears to effectively average over the heterogeneities in host susceptibility, presumably responsible for the outliers at high and low doses present in the individually housed experimental model (Figure 1). This averaging is at the expense of effectively reducing the number of experimental replicates to the number of groups rather than the number of individual birds.

The beta-Poisson model has been favored in previous studies of Campylobacter spp. dose-response in broilers (Chen et al., 2006) primarily on pragmatic grounds. We have demonstrated that the extent of host heterogeneity in the ability to be colonized by Campylobacter spp., as demonstrated by the slope of the observed dose-response relationship, can be obscured by transmission between co-housed birds. As evidenced by our analysis of the individually housed chicks, estimates of CD$_{50}$ under the single-hit model are particularly sensitive to the effect of outliers due to the fixed width of the dose-response relationship of $-2 \log_{10}$. The added flexibility of the beta-Poisson model eliminated this effect providing consistent estimates of the CD$_{50}$ for C. jejuni RM1221 under both housing models.

Elimination of bird-to-bird transmission, by co-housing, is now a viable strategy to reduce the colonization and shedding of Campylobacter jejuni to reduce the colonization and shedding of Campylobacter jejuni. However, the method is not without its limitations. The single-hit dose-response model, which is assumed to be valid under co-housing, may not reflect the true biological mechanism of transmission. Further studies are needed to better understand the role of co-housing in reducing the transmission of Campylobacter jejuni.

**REFERENCES**


