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Campylobacter epidemiology from breeders to their progeny in Eastern Spain.

S. Ingresa-Capaccioni*, E. Jiménez-Trigos*, F. Marco-Jiménez†, P. Catalá‡, S. Vega*, C. Marin*[1]

*Instituto de Ciencias Biomédicas. Departamento de Producción Animal, Sanidad Animal y Ciencia y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad CEU-Cardenal Herrera, C/Tirant Lo Blanc 7, 46115 Alfara del Patriarca, Valencia, Spain.

†Instituto de Ciencia y Tecnología Animal, Universidad Politécnica de Valencia, C/ Camino de Vera s/n, 46022, Valencia, Spain.

‡Centro de Calidad Avícola y Alimentación Animal de la Comunidad Valenciana (CECAV), C/ Nules 16, 12539 Alquerías del Niño Perdido, Castellón, Spain.

[1]Corresponding author: clara.marin@uch.ceu.es

Prof. PhD MVS Clara Marín Orenga. Instituto de Ciencias Biomédicas. Departamento de Producción Animal, Sanidad Animal y Ciencia y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad CEU-Cardenal Herrera, C/Tirant Lo Blanc 7, 46115 Alfara del Patriarca, Valencia, Spain. Tel.: +34 96 1369000 | Ext. 66107.

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ABSTRACT

While horizontal transmission is a route clearly linked to the spread of *Campylobacter* at farm level, few studies support the transmission of *Campylobacter* spp. from breeder flocks to their offspring. Thus, the present study was carried out to investigate the possibility of vertical transmission. Breeders were monitored from the time of housing day-old chicks, then throughout the laying period (0 to 60 weeks) and throughout their progeny (broiler fattening, 1 to 42 days) until slaughter. All samples were analyzed according with official method ISO 10272:2006. Results revealed that on breeder farms, *Campylobacter* isolation started from week 16 and reached its peak at week 26, with 57.0% and 93.2% of positive birds, respectively. After this point, the rate of positive birds decreased slightly to 86.0% at 60 weeks. However, in broiler production all day-old chicks were found negative for *Campylobacter* spp, and the bacteria was first isolated at day 14 of age (5.0%), with a significant increase in detection during the fattening period with 62% of *Campylobacter* positive animals at the end of the production cycle. Moreover, non-positive sample was determined from environmental sources. These results could be explained because *Campylobacter* may be in a low concentration or in a non-culturable form, as there were several studies that successfully detected *Campylobacter* DNA, but failed to culture. This form can survive in the environment and infect successive flocks; consequently, further studies are needed to develop more modern, practical, cost-effective and suitable techniques for routine diagnosis.

Key words: *Campylobacter*, hen, broiler, epidemiology.

INTRODUCTION

Campylobacteriosis is the most frequently reported zoonosis in the EU and one of the most common causes of diarrheal illness in the United States, and the incidence appears to be increasing (CDC, 2011; EFSA, 2014). The European Food Safety Authority (EFSA) reported a total of 214,268 cases of human campylobacteriosis in 2012, and the Center for Disease Control (CDC) estimates that each year 845,024 cases of human campylobacteriosis occur in the United States (Scallan et al., 2011). Typical symptoms include watery and/or bloody diarrhea, abdominal pain, fever, nausea, headache, and vomiting (Altekruse et al., 1999). The illness is typically self-limiting and usually resolves in around one week; however, severe illness and long-term complications, such as arthritis, septicemia and Guillain-Barre syndrome, a demyelinating disorder, which causes acute neuromuscular paralysis, respiratory muscle compromise and death, sometimes occur (Nachamkin et al., 1998).

Poultry and poultry products are considered the main source of human campylobacteriosis (EFSA, 2014) and the majority of infections result from consumption of undercooked poultry or other food products cross-contaminated with raw poultry meat during food preparation (Jacobs-Reitsma, 2000; Corry and Atabay, 2001). Specifically, in the European context broiler meat may account for 20-30% of human campylobacteriosis cases, while 50-80% may be attributed to the chicken reservoir as a whole (EFSA, 2014).

The epidemiology of *Campylobacter* in poultry production is still not completely understood (Cox et al., 2012). For more than a decade there has been a major debate on whether vertical or horizontal transmissions are responsible for the introduction of *Campylobacter* into flocks (Sahin et al., 2002; Cox et al., 2012). Clearly, horizontal transmission has been identified through different sources, while the vertical transmission from parent flocks and their progeny still remains unclear (Cox et al.,

2012; Agunos et al., 2014). *Campylobacter* has been detected from both oviducts of laying hens (Jacobs-Reitsma, 1997; Camarda et al., 2000; Buhr et al., 2002; Hiett et al., 2002b; Cox et al., 2005) and semen of commercial broiler breeder roosters (Cox et al., 2002b; Hiett et al., 2003a). Another possible path for introduction of *Campylobacter* into chicken flocks is transmission from the hen through the egg to the chick (Newell and Fearnley, 2003). *Campylobacter* has been isolated from the inner shell and membranes of eggs, revealing a possible route of exposure for hatching chickens (Doyle, 1984; Shane et al., 1986; Allen and Griffiths, 2001). Cox et al. (2002a) found identical ribotypes and *flaA* short-variable-region alleles in a commercial broiler breeder flock and its progeny broiler flock. In addition, other studies have shown the presence of amplifiable *Campylobacter* DNA in hatchery fluff, intestinal tracts of developing embryos and newly hatched chicks, which support the molecular evidence that *Campylobacter* can be present in chicks before they are delivered to the farm (Chuma et al., 1994, 1997b; Hiett et al., 2002a: 2003b; Idris et al., 2006). However, other studies have not supported the hypothesis that vertical transmission makes a significant contribution to the dissemination of *Campylobacter* on poultry farms (Sahin et al., 2003b; Bull et al., 2006; Callicott et al., 2006; O'Mahony et al., 2011).

In this context, the aim of the present study was to assess *Campylobacter* spp isolation from breeders and throughout their progeny (broiler) to determine the importance of vertical transmission.

MATERIALS AND METHODS

The Ethics and Animal Welfare Committee of the Universidad CEU Cardenal Herrera approved this study. All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette).

Study sample

From January 2012 to August 2013, a longitudinal and vertical study of the whole poultry production cycle was carried out in the Valencian Region (Eastern Spain). Breeder birds were monitored from the time just before placing the day-old chicks in the houses (rearing), then throughout the laying and fattening period (broiler) until slaughter. Samples from 7 breeder flocks and 21 broiler flocks were analyzed for *Campylobacter*. The sample collection scheme is shown in Figure 1.

Environmental sample collection

To assess the *Campylobacter* status of the houses, at the beginning and at the end of the production period (breeder and fattening), environmental samples (water, dust, surfaces, feed and farming boots) were taken. Each sample was taken using different strategies. First, house surfaces and farmer boot samples were taken with sterile wet gauze pads (AES laboratories, Bruz Cedex, France). Feed samples were collected from the truck and feeders (about 500 g) and water was sampled from the tank and final dispenser lines (500 mL). Then, 100 g of dust (250 mL) were collected from different points of the house. The sample was homogenized in the laboratory and 25 g were analyzed.

Sample collection in breeder flocks (parents)

A total of seven breeder flocks were visited and sampled at different times throughout productive life (rearing period 0-20 weeks and laying period 20-60 weeks). Each flock was located on one farm. A total of 12 and 25 houses were sampled during the rearing and laying period, respectively. The first visit occurred just before placing the day-old chicks in the houses. To assess *Campylobacter* status of the animals upon the arrival at the farm, ten birds were randomly selected and euthanized by cervical dislocation. After necropsy, the pair of ceca were removed and placed in an individual sterile jar. Ceca samples were pooled into a composite sample for the detection of *Campylobacter*. During rearing period, each flock was sampled collecting 10 cloacal swabs at 4, 8, 12,

16, and 20 weeks. Cloacal samples were taken individually using sterile swabs (Cary Blair transport sterile swabs, DELTALAB®). At the end of the rearing period (20 weeks), the animals were transported to the laying farms. To assess the *Campylobacter* status of the slaughter truck, when the truck arrived at the farm, containers and platform were sampled with sterile wet gauze pads with disinfectant neutralizer (AES Laboratories, Bruz Cedex, France). Then, after transport, birds' cloacal samples were also taken. Finally, during the laying period, 10 cloacal samples per flock were also collected at 26 weeks (onset of laying period), 31 weeks (peak of lay), 48 weeks (spiking), and 60 weeks (end of laying period).

Sample collection in broiler flocks (offspring)

In this study, a total of 21 broiler flocks corresponding to the progeny of the breeder flocks during productive life were evaluated. Each flock was sampled just before placing day-old chicks (day 1), collecting the pair of ceca, as described for the breeder flocks. Then, cloacal samples were collected at weekly intervals during the fattening period (7, 14, 21, 28, 35 and 42 days), when 10 cloacal swabs were collected from each flock, as described previously.

Sample collection at slaughter

All broiler flocks were monitored at the slaughterhouse. For this purpose, three carcasses from each broiler flock were tested. A neck skin sample was collected from each carcass after chilling.

Campylobacter isolation

The samples collected were tested by direct culture (Vidal et al., 2013) and enrichment culture based on Official method ISO 10272:2006 (Annex E) (ISO, 2006), except for cloacal swabs, which were only examined by direct culture. The LOD, for the ISO method in the different kind of samples, is less than 100 CFU/sample, around 50 CFU/sample *C. jejuni* and 65 CFU/sample *C. coli*.

Water samples were processed by mixing 25 mL sample with 225 mL PBS; this was then homogenized by stirring. Feed samples were processed by mixing 25 g sample with 225 mL PBS; this was then and homogenized for 60 s using a filter Stomacher bag (Separator 400; Seward, West Sussex, United Kingdom) and a Stomacher (Stomacher 400; Seward, West Sussex, United Kingdom). Surface and boot samples were processed by mixing each sterile wet gauze pad with 50 mL PBS; this was then homogenized. Sock swabs were mixed with 100 mL PBS; this was then homogenized. The feces samples were processed mixing 25 g from each jar with 225 mL PBS; this was then homogenized. The cecal samples were processed and cultured as described by Rodgers et al. (2010). Briefly, a pooled cecal sample was created by homogenizing 0.02 g cecal content from one cecum from each of the 10 birds collected from the house into 2 mL PBS. From all sample types, 10 μL aliquots of each suspension were plated onto modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid, Dardilly, France) and Preston agar (CM0689, Oxoid, Dardilly, France). Then the samples were incubated at 41.5 ± 1◦C in a microaerobic atmosphere (84% N2, 10% CO2, 6% O2) for 48 h, except for the cloacal swabs, which were directly plated onto mCCDA and Preston agar and incubated as previously described. Moreover, samples were pre-enriched in 1:10 vol/vol Bolton broth (CM0983, Oxoid, Dardilly, France) and then pre-incubated at 37 ± 1oC for 5 ± 1 h. Finally, the pre-enriched broth was incubated at 41.5 ± 1◦C for 43 ± 1 h. Afterward, 100 μL sample was cultured on the 2 selective agar plates (mCCDA and Preston agar) and incubated as described above.

Plates were examined for grey, flat, irregular and spreading colonies typical of *Campylobacter*. *Campylobacter*-like colonies were purified on blood agar and identified to species level on the basis of standard procedures (ISO, 2006). One putative colony was subcultured from each plate onto sheep blood agar for confirmation as *Campylobacter* spp. *Campylobacter* confirmation was performed by a mobility test

using a dark field microscope, by oxidase and catalase biochemical test and by streaking at different temperatures and atmospheres on Columbia blood agar (AES Laboratories®, Bruz Cedex, France), because *Campylobacter* will fail to grow at 25ºC in micro-aerobic atmosphere (84% N2, 10% CO2 and 6% O2) conditions and at 41,5ºC in aerobic conditions. Finally, characterization of the bacterial species was done by hippurate hydrolysis test.

Statistical analyses

A generalized linear model with a binomial probability distribution and a logit link function was used to compare the isolation of *Campylobacter* in chickens throughout the productive life in breeders (0, 4, 8, 12, 16, 20, 26, 31, 48 and 60 weeks of productive life) and throughout the fattening period in broilers *(*1, 7, 14, 21, 28, 35 and 42 days of rearing period). The farm was incorporated in the model as a repeated measurement factor. For this analysis, the error was designated as having a binomial distribution and the probit link function was used. Binomial data for each sample were assigned a 1 if *Campylobacter* was isolated from poultry or a 0 if not. A P value of less than 0.05 was considered to indicate a statistically significant difference. Data are presented as least squares means ± standard error of the least squares means. All statistical analyses were carried out using a commercially available software program (SPSS 16.0 software package; SPSS Inc., Chicago, Illinois, USA, 2002).

RESULTS

Environmental samples

A total of 580 environmental samplings were conducted in the breeder (n=370) and broiler flocks (n=210). No *Campylobacter* spp. was isolated in any of the environmental samples from the breeder and broiler flocks.

Breeders (parents)

During the study period, 1,040 samples out of 1,780 were found positive for *Campylobacter*. *Campylobacter* was not isolated in any of the day-old chicks sampled. The bacterium was first isolated at 16 weeks of the rearing period (57.0%, Figure 2). The bacteria isolation from individual breeders was homogeneous until week 26 (laying period), with a peak of 93.2% (Figure 2). After week 26, a slight decrease in *Campylobacter* isolation occurred (Figure 2). Moreover, *Campylobacter jejuni* was the most commonly identified species (67%, 77.0%, 83.0%, 88.0%, 78.0% and 86.0%, at 16, 20, 26, 31, 48 and 60 weeks, respectively). All breeder flocks (n=7) were negative for *Campylobacter* at the beginning of the rearing period. However, at the end of rearing and the beginning of the laying period four of the seven breeder flocks studied were found positive for *Campylobacter*. All breeder flocks were positive by the end of the laying period (n=7).

In addition, no differences were found between the individual positive rates before and after transport of the animals from the breeder farms to the breeder laying farms at the end of the rearing period. The percentage of positive animals were 54.0% and 59.0%, before loading and after transport, respectively (Figure 2). All samples collected from transport trucks were negative for *Campylobacter* spp.

Broiler flocks (offspring)

No day-old chick sampled was found positive for *Campylobacter*. During the fattening period, 329 samples out of 1,260 were positive for the bacterium. *Campylobacter* isolation of chickens differed significantly depending on the day of the fattening period (Figure 3). *Campylobacter* was first isolated in chickens at day 14 of age (5.0%, Figure 3) and the isolation increased significantly throughout the fattening period, with the highest rate at the end of fattening (62.0%, Figure 3). *Campylobacter jejuni* was the most commonly identified species (100.0%, 87.0%, 90.0%, 75.0% and 67.0%, at 14, 21,

28, 35 and 42 days of rearing period, respectively). All broiler flocks (n=21) were declared negative for *Campylobacter* at day 0 of rearing. However, at day 42 almost all were positive for the bacteria (n=20).

Slaughterhouse

At slaughter, 3 broiler carcass samples from each flock were evaluated. A total of 52 samples out of 63 were positive for *Campylobacter* (82.5%). *Campylobacter jejuni* was the most commonly identified species (75.0%).

DISCUSSION

Campylobacter contaminated broiler meat is a public health concern worldwide (EFSA, 2015). Therefore, reducing the prevalence of *Campylobacter* at the primary production level is expected to result in a low concentration or absence of *Campylobacter* on the final product, and consequently in a reduction of human exposure (Wagenaar et al., 2006; EFSA, 2010). Previously conducted reviews on *Campylobacter* sources for poultry summarize evidence for horizontal transfer of *Campylobacter* in the poultry industry (Adkin et al., 2006; EFSA, 2010; Newell et al., 2011; Cox et al., 2012; Agunos et al., 2014). Nevertheless, few studies support the possibility of vertical transmission (Adkin et al., 2006). Thus, the present study was carried out to investigate the possibility of vertical transmission.

Our earlier experiment (breeder stage) revealed that *Campylobacter* isolation started from week 16 and reached its peak at week 26, with 57.0% and 93.2%, respectively. After this point, the rate of *Campylobacter* isolation decreased slightly to 86.0% at 60 weeks. In contrast, broilers flocks were determined as a positive only 2 to 3 weeks after the chick placement in a broiler house (Sahin et al., 2002; Jacobs-Reitsma et al., 1995; Evans and Sayers, 2000; Newell and Wagenaar, 2000; Shreeve et al., 2000). This may be because breeder farms maintain high levels of biosecurity and employed standard

257 vaccine handling practices, while commercial broiler farms maintain basic biosecurity
258 practices (Perez-Boto et al., 2012; Mutinda et al., 2014). In addition, an inadequate
259 cleaning and disinfection and a short downtime of the broiler houses between flocks
260 have both been identified as a major source of *Campylobacter* colonization of the birds
261 (Rivoal et al., 2005; Bull et al., 2006, Messens et al., 2009; Allen et al., 2011;).
262 However, all environmental samples investigated in this study were found negative for
263 *Campylobacter*.
264 The results of our survey indicate that *Campylobacter* isolation is elevated during their
265 egg-productive lives (from 26 to 60 weeks). Although vertical transmission has often
266 been considered a rare event (Sahin et al., 2003b; Bull et al., 2006; Callicott et al., 2006;
267 O'Mahony et al., 2011), it has been suggested that one possible path for introduction of
268 *Campylobacter* into broiler flocks could be from the hen through the egg to the chick
269 (Newell and Fearnley, 2003). Some studies have showed that feces can easily
270 contaminate the surface of an egg, as the egg and feces both pass through the cloaca
271 (Buhr et al., 2002; Cox et al., 2012). Then, when the chick emerges it can ingest
272 *Campylobacter* and become colonized, resulting in an infected broiler flock as only a
273 few susceptible birds are sufficient to result in flock-level colonization by the end of
274 grow out (Ring et al., 2005; Katsma et al., 2007; Cox et al., 2012). This is expected to
275 occur in a relatively small number of birds, making it difficult to detect *Campylobacter*
276 at the beginning of rearing (Cox et al., 2012). Despite of these findings, in this study, all
277 day-old chicks tested were free of *Campylobacter*, according to the results of previous
278 studies (Newell and Fearnley, 2003; Bull et al., 2006; Callicott et al., 2006), and the
279 onset of colonization in broilers was not detected until day 14 of rearing.
280 Several hypotheses have been put forward to explain researchers' difficulty to isolate
281 *Campylobacter* during the first two weeks of placement. First, protective maternal
282 antibody effects delay *Campylobacter* colonization (Sahin et al., 2002, 2003a). Second,

283 the type of sample may be important, for example, *Campylobacter* may not be present
284 in the cecal or fecal samples during early rearing because it is still colonizing the small
285 intestine (Idris et al., 2006; Hiett et al., 2013). Third, different isolation techniques have
286 highly variable sensitivity that may affect results if *Campylobacter* concentration is
287 below the detection limits (Chuma et al., 1997a). Because of the inherently low number
288 of cells in eggs/eggshells, embryos, yolk sac, and neonatal intestines, enhanced recovery
289 techniques (e.g., combining membrane filtration and enrichment) need to be explored to
290 improve our detection limits in these samples (Jokinen et al., 2012). Fourth,
291 *Campylobacter* may be in a non-culturable form as there were several studies that
292 successfully detected *Campylobacter* DNA, but failed to culture (Chuma et al.,
293 1994:1997b; Sahin et al., 2002). Thus, there is a need to explore the use of a more
294 reliable molecular technique for detecting viable or "potentially infectious units" of
295 *Campylobacter* (Kruger et al., 2014) from hatchery and chick samples (Agunos et al.,
296 2014).
297 In this study, we sampled breeder (parent) and broiler flocks (offspring) to evaluate the
298 importance of vertical transmission. We found no evidence of transmission from
299 vertical and environmental sources. However, the inability to culture *Campylobacter*
300 when its concentration is below the detection limits (Kruger et al., 2014), or when the
301 bacteria is present in a non-culturable form (Chuma et al., 1994:1997b; Sahin et al.,
302 2002), can be considered a constraint to knowing the ecology of *Campylobacter* and
303 therefore the exact routes of transmission. While studies do not definitively rule out the
304 detection problems and an accepted standard method will be developed for the detection
305 and isolation of *Campylobacter* spp. at farm level, no standard measure may be
306 successfully implemented in broiler production and therefore, from a public health point
307 of view, strategies to reduce the number of human campylobacteriosis cases will not be
308 efficient.

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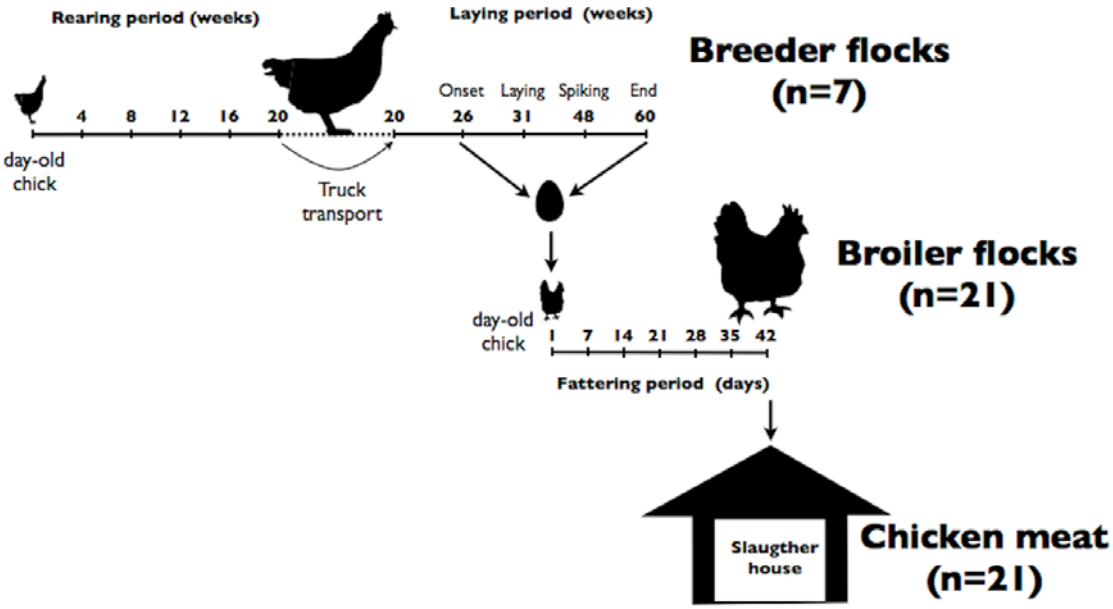
Figure 1. Schematic illustration of the samples collection to determine vertical transmission of *Campylobacter* passage from breeder hens to broiler progeny. For the breeder flocks, during rearing period, samples were collected at 0, 4, 8, 12, 16, and 20 weeks and during laying period samples were collected at 26, 31, 48 and 60 weeks. For the broiler flocks, during the fattening period, samples were collected at 7, 14, 21, 28, 35 and 42 days.



488 **Figure 2.** *Campylobacter* isolation during the breeding period (rearing and laying
489 period). Each animal was sampled from 0 to 60 weeks of age. [a,b,c,d,e]Different
490 superscripts represent significant differences ($P < 0.05$). Data are presented as least
491 squares means ± standard error of the least squares means.
492

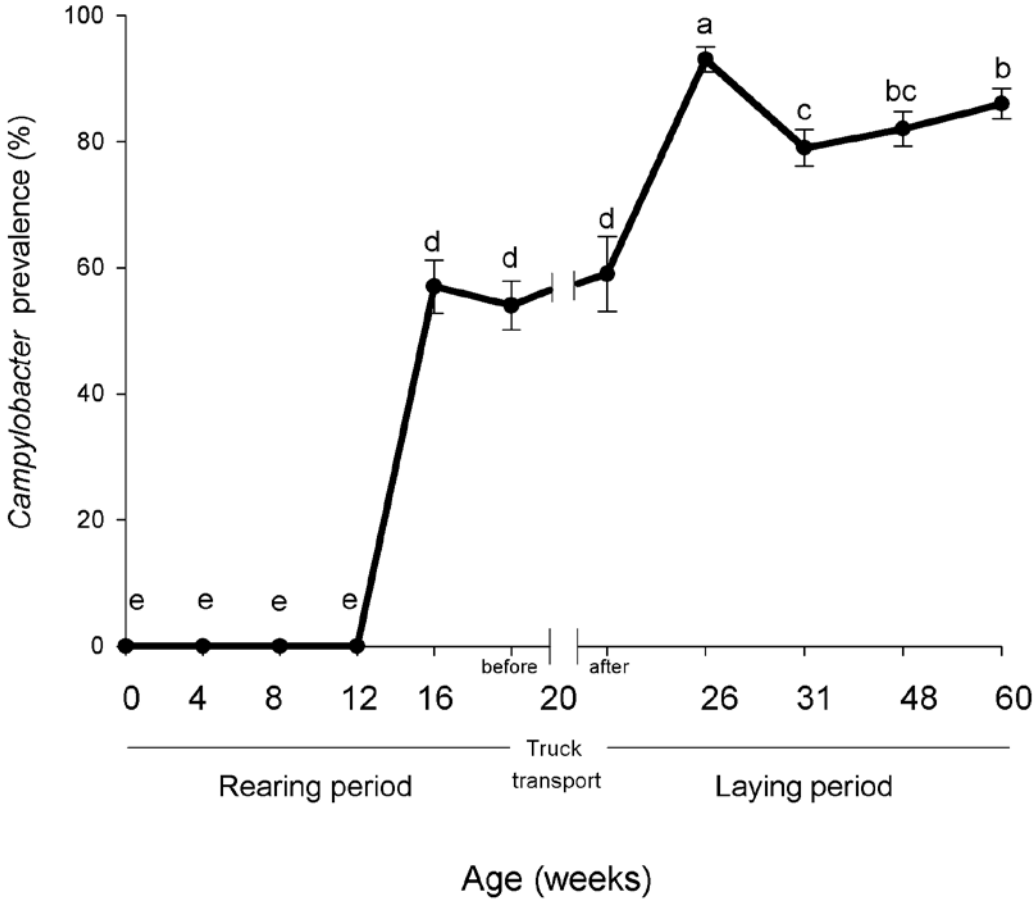


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Figure 3. *Campylobacter* isolation during fattening period. Each animal was sampled just before placing day-old chicks (day 1) and at weekly intervals until slaughter day. [a,b,c,d,e,f] Data with uncommon letters are different ($P < 0.05$). Data are presented as least squares means ± standard error of the least squares means.