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Protecting broilers against *Campylobacter* infection by preventing direct contact between farm staff and broilers

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ABSTRACT

The objective of this study was to test the hypothesis that farm staff are the primary vector of *Campylobacter* transmission into broiler flocks. On 3 different farms and 5 different flocks (3 flocks on farm 1 and 1 flock on each of farms 2 and 3) a small section of the broiler house (3 × 2 m (farm 1) and 1 m × 1 m (farms 2 and 3)) was sectioned off using Perspex or plastic sheeting. This 'biosecure cube' (BC) was populated with 25–125 chicks (test birds), a small subset of the general population of up to 30,000 (control) birds in the broiler house. The BC area incorporated the water and feed-lines thus the test and control birds had access to the same feed, water and air. However, unlike in the general broiler house, the farm staff had no direct access to this sub-population. Dead birds were aseptically removed by the researchers. The birds were tested for *Campylobacter* (faecal and/or caecal samples), on the day of chick arrival and every 7 days thereafter. In farm 1-flock 1 the general broiler population was *Campylobacter*-positive after 21 days but the test birds remained negative until day 35. The general broiler population in the other 4 flocks were *Campylobacter* positive as early as day 14, but in all cases the test birds remained negative. Moreover BC broilers were significantly ($P < 0.05$) heavier than the control birds (400 g on average), at first thinning. It was therefore concluded that preventing direct contact between the farm staff and the broilers prevents *Campylobacter* infection in broilers.

1. Introduction

Campylobacter spp. are microaerophilic, fastidious, zoonotic pathogenic organisms (Silva et al., 2011), which, although ubiquitous in the environment, preferentially colonise farmed poultry (Newell & Fearnley, 2003). Campylobacteriosis is the most common gastroenteritis in the developed world and its incidence in the EU is conservatively estimated at 9 million cases per annum costing €2.4 billion (EFSA, 2011). Poultry are the primary source accounting for 50–80% of cases (EFSA, 2011). Approximately 83% of the 70 million broilers produced in Ireland each year are infected with *Campylobacter* (EFSA, 2010a).

Multiple sources of *Campylobacter* have been identified on broiler farms, including flies (Hald et al., 2004; Hald, Sommer, & Skovgård, 2007), rodents (Meerburg, Jacobs-Reitsma, Wagenaar, & Kijlstra, 2006), water (Pérez-Boto et al., 2010), adjacent livestock (Doyle & Erickson, 2006), pets (Whiley, van den Akker, Giglio, & Bentham, 2013), and dirty equipment (Agunos, Waddell, Léger, & Taboada, 2014). Thus preventing *Campylobacter* ingress into a poultry house is reliant on good biosecurity, including the application of foot dips, an ante-room with clean and dirty zones, effective terminal hygiene, house specific footwear, hand washing facilities, effective litter management, equipment hygiene and rodent control activities (Bord Bia, 2008). However, even when these are in place, flocks are still regularly infected, primarily due to a failure of farm staff to consistently apply biosecurity procedures (Newell et al., 2011), resulting in farm staff (and other personnel) serving as a major vehicle of *Campylobacter* carriage into the broiler house (Allen et al., 2008).

The objective of this study was therefore to test the hypothesis that farm staff are the primary source of *Campylobacter* transmission into broiler flocks and preventing direct contact between them and the birds would protect the flock against *Campylobacter* infection.

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2. Method and materials

2.1. Description of the farms used in the study

This study was initially undertaken on one farm (farm 1) using 3 different flocks (flocks 1, 2 and 3) at different times. It was then extended to include 2 additional farms (farms 2 and 3) using one flock per farm (flocks 4 and 5). There were approximately 33,000 birds in flocks 1 to 3, 22,000 in flock 4 and 35,000 in flock 5. The broiler farms all used fan based controlled ventilation and each had between 2 and 5 broiler houses in close proximity on a single site with a tarmac apron. Thinning or partial depopulation of flocks was carried out once in each flock, typically between day 32 and 37, at which point the experiment was terminated.

2.2. Description of biosecure cube used in the study (flocks 1–3)

The 'biosecure cube' (BC) used on farm 1 (flocks 1 to 3), consisted of 6 mm thick clear polycarbonate sheets (Goldstar Plastics, Dublin) on all 4 sides supported by four 1 m high wooden columns (Wood Workers, Dublin) on each corner (Picture 1). The total internal floor area was 6 m^2. Four slits in the polycarbonate sheets (50 cm high×8 cm wide), lined with industrial 50 mm thick bristle strips (Ibex Industrial Brushes, UK), allowed the feeder and drinker lines to run through the unit. The top of the unit was initially covered with a fly screen mesh (flock 1 only) (PetScreenMesh®, Modern Flyscreens, Tullamore, Offaly, Ireland) with a pore size of 0.914 mm, but this had to be removed after approximately 10 days as it became clogged with dust. Exactly 125 'test' birds were reared within this BC to ensure the stocking density was the same as that in the rest of the broiler house. Farm staff were instructed not to enter or interfere with the unit under any circumstances. If equipment failed or a fatality occurred the researchers were informed and carried out the necessary actions.

2.3. Description of 'biosecure cube' used for flocks 4 &5

The 'biosecure cube' (BC) unit used on farms 2 and 3 (flocks 4 and 5), consisted of clear polyethylene sheets (B&Q, Dublin) tacked onto a wooden frame consisting of 4 sides, each made from 4 × 1 m wooden slats (40 cm × 20 cm) (Picture 2). The total internal area was 1 m^2. The four slits in the polyethylene sheets which accommodated the feeder and drinker lines were secured with Universal tape. No fly screen was applied to these units. Stocking density placed inside the BC on each occasion was equivalent to that in the rest of the house, with 25 birds placed inside each time. As per above, farm staff were instructed not to enter or interfere with the unit under any circumstances.

2.4. Sample collection

Samples were collected from each flock on the day of chick arrival and every 7 days during the broiler rearing period. These included; [1] 40 air Samples (tested for *Campylobacter* and Total Viable Count's (TVC)); [2] 100 faecal samples (10 pooled samples each containing 10 fresh faecal samples, collected directly from the broiler house floor; [3] 10 faecal samples collected from the floor of the BC; [4] 3 × 50 g of feed from the feed auger supplying the feed line that included the BC; [5] 3 L of the broiler house water supply and [6] 10 caecal samples, each collected once per week from 10 randomly selected 'control' birds. Once the flock tested positive for *Campylobacter* (or the flock reached 21 days), caecal testing was extended to include the birds within the BC (10 per week from flocks 1 to 3 and 5 per week from flocks 4 and 5).

Air samples were taken using a AI3P Air Ideal 3P unit (Biomérieux, France). Ten litres were sampled for each air plate. Plate count agar (PCA, CM0325B Oxoid, Cambridge, UK) was used to collect total viable counts and modified *Campylobacter* blood free selective agar (mCCDA, CM0739b, Oxoid, Cambridge, UK) supplemented with cefoperazone and amphotericin (SR0155E, CCDA selective supplement, Oxoid, Cambridge, UK) plates were used to detect *Campylobacter*.

Water samples were collected using 3 × 1 L water sampling bottles (VWR International Ltd, Dublin). The tap was sprayed with 70% ethanol, flamed and the water allowed to run for 5 min before water samples were taken.

Samples were taken up to first thin which normally occurred between 32 and 37 days. Samples were transported to the laboratory at 4 °C in a cool box and processed within 24 h.

Bird weights were obtained by the poultry veterinarian *post mortem* on days when the birds were removed for caecal testing and/or from day 21 onwards. Post day 21 is considered to be the 'developmental phase' for broilers and a minimum of 25% of the birds were sampled for weights on days 21, 28 and 35.

2.5. Campylobacter isolation

To detect *Campylobacter*, samples were both direct plated and enriched according to the Horizontal Method for Detection and Enumeration of *Campylobacter* spp. (ISO 10,272, 2006). Composite faecal samples were prepared by adding 25 g to 225 mL of Bolton broth (CM983B, Oxoid, Cambridge, UK) supplemented with 5% lysed horse blood (SR048C, Lennox, Dublin) and a selective supplement containing cefoperazone, vancomycin, trimethoprim and cycloheximide (SR183E, Bolton broth supplement, Oxoid, Cambridge, UK), to give a 1:10 dilution and stomached for 30s. After mixing, serial dilutions were prepared using maximum recovery diluent (MRD) (CM0733B Oxoid, Cambridge, UK) and 100 μL aliquots were plated out on modified mCCDA for each composite sample. Sample inoculated broths were also enriched at 37 °C for 5 h followed by 42 °C for 48 h under microaerobic conditions using Anaero Jars (AG0025A, Fannin, Dublin) with Campygen atmosphere generation kits (CN025A, Oxoid, Cambridge, UK). Samples were plated out on mCCDA following incubation.

Caecal samples were both direct plated and enriched as per ISO 10,272: 2006. Briefly, 1 g of caecal material was added to 9 ml of Bolton Broth and vortexed. Serial dilutions were prepared in MRD, and 100 μL volumes plated out on mCCDA. The remaining broths containing caecal contents were enriched by incubating under microaerobic conditions as above at 37 °C for 5 h followed by 42 °C for 48 h. After incubation, samples were plated out on mCCDA.

Air plates were incubated as follows; PCA air plates were inverted and incubated at 30 °C for 48 h, mCCDA were inverted and incubated under microaerobic conditions at 37 °C for 5 h followed by 42 °C for 48 h. After incubation plates were inspected for colonies.

Water samples (3 L) were initially filtered through 0.45 μm (Millipore, MA, USA) membranes. Filters were then aseptically transferred to 100 mL Bolton enrichment broth and incubated at 37 °C for 5 h followed by 42 °C for 48 h. After enrichment the samples were plated out on mCCDA.

Feed Samples were analysed by adding 10 g to 90 mL Bolton Broth followed by vortexing for 30s. The samples were then incubated as previously described and plated out on mCCDA after enrichment.

2.6. Campylobacter identification

All presumptive *Campylobacter* isolates were confirmed initially using standard biochemical tests; Gram reaction (3% [w/v] KOH,

Sigma Aldrich, Ireland), Oxidase test (Oxoid, Cambridge, UK) and L-ala test (Sigma Aldrich, Arklow, Wicklow, Ireland) followed by streaking on Campy Food ID chromogenic agar (Biomerieux, Durham, NC). After biochemical and chromogenic testing, the isolates were further confirmed using Real Time PCR. All isolates were stored at −80 °C on Protect cryobeads (TSC, Heywood, Lancashire, UK) in 80% glycerol.

2.7. Further confirmation by Real-Time PCR

2.7.1. DNA preparation

All *Campylobacter* isolates recovered from samples were grown on Columbia blood agar plates (Oxoid, Cambridge, UK) supplemented with 5% Lysed horse blood at 42 °C for 48 h. A loopful of cells from a colony on each plate was added to 20 μL proteinase k and 180 μL Buffer ATL and extracted using the DNeasy blood and tissue kit following the manufacturer's instructions (Qiagen, Manchester, UK).

2.7.2. Real-Time PCR assay

A Real-Time PCR assay (C16S LvI) was used to identify *Campylobacter* isolates to genus level by amplification of the 16SrRNA gene (De Boer et al., 2013). Each 10 μL reaction mixture contained: 0.4 μL forward primer, 0.4 μL reverse primer, 0.2 μL probe, 5 μL Roche Lightcycler master mix (Roche, City west, Dublin), 2 μL deionised PCR quality water (Sigma Aldrich, Ireland) and 2 μL DNA.

The following PCR conditions were used: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15s, 60 °C for 1 min and an extension step of 40 °C for 30 min. Each reaction contained a positive control of NCTC 11,168 *C. jejuni* and a negative control of *Escherichia coli 3514D* from the Teagasc culture collection as well as a non-template DNA control. Isolates were deemed positive if their amplification occurred between cycles 14–35.

2.8. Statistical analysis

Statistical comparison of final slaughter weights (day 35) was performed in GENSTAT by Anova ver. 14.1 (VSN International Ltd., Hemel, Hempstead, UK). Significance was determined at the 5% ($P < 0.05$ level).

3. Results

Based on *Campylobacter* detection in broiler faeces, flock 1 (control birds) were *Campylobacter* positive after 21 days (4.5 $\log_{10}$ CFU/g) (Table 1). The test birds in the biosecure cube remained *Campylobacter* negative until day 35 (1.5 $\log_{10}$ CFU/g). Two subsequent flocks (2 and 3) on the same farm also recorded *Campylobacter* positive faeces after 21 days (5.1 and 3.5 $\log_{10}$ CFU/g, respectively) but the test birds in the biosecure cube remained negative. This pattern was repeated on 2 other farms, with flocks 4 and 5 control birds infected with *Campylobacter* after 35 and 14 days (positive after enrichment), respectively while the birds within the biosecure cube remained *Campylobacter* free. Similar results were obtained with the caecal samples (Table 2). With the exception of flock 1, which turned positive at day 35, all of the test birds remained *Campylobacter* negative while the control birds in the general population were infected with *Campylobacter* by days 14, 21 and 35. Interestingly, the birds in the biosecure cube were protected despite receiving the same feed and water and breathing the same air. While all feed and water samples tested negative throughout these experiments, the air in the flock 1, 2 and 5 broiler houses was contaminated with *Campylobacter* as early as day 14 and reached levels as high as 4.3 $\log_{10}$ CFU/m^3 (Table 3). Average air TVC counts at the beginning of the rearing periods were 4.2 $\log_{10}$ CFU/m^3 which increased to 5.5 $\log_{10}$ CFU/m^3 at the end of the rearing cycle (data not shown). The highest air TVC concentrations were recorded in flock 5 (5.93 $\log_{10}$ CFU/m^3) on day 35.

Weight gain in the test and control birds was also recorded. From day 21 to day 35 (the developmental phase) the test birds consistently showed greater weight gain than birds in the general population (Table 4) so that by day 35 the test birds were significantly heavier ($P < 0.05$) (on average 400 g) than the control equivalents.

4. Discussion

In the absence of other control options, keeping broiler flocks *Campylobacter* free is reliant on effective biosecurity. However, the effectiveness of biosecurity depends on consistency of application and non-compliance is common in the poultry sector. Racicot, Venne, Durivage, and Vaillancourt (2011), in an observational study in Canada, reported 44 different biosecurity breaches from 883 visits by 102 different individuals on broiler farms (an average of 4 non-compliances per visit). Approximately 61% of errors were related to cross-contamination of clean and contaminated areas, 14% to improper procedures for footwear, 11% to inadequate hand washing and 7% to unclean overalls. This behaviour may be attributed to a lack of; training, understanding of the consequences, time, incentives, motivation as well as apathy and denial (Vaillancourt & Carver, 1998); a combination of which can be impossible to remediate.

Moreover, the pattern of *Campylobacter* contamination on broiler farms will also mitigate against effective biosecurity. Once a flock is *Campylobacter* positive, these bacteria are readily spread

Table 1
Campylobacter faecal counts in the flocks (C) and the BC (T) on the farms tested.

Campylobacter count ($\log_{10}$ CFU/g)										
Time (Days)	Farm 1						Farm 2		Farm 3	
	Flock 1		Flock 2		Flock 3		Flock 4		Flock 5	
	T	C	T	C	T	C	T	C	T	C
0	N.D[a]	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
7	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
14	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	PAE[c]
21	N.D	4.5	N.D	5.1	N.D	3.5	N.D	N.D	N.D	4.8
28	N.D	7.2	N.D	6.5	N.D	6.7	N.D	N.D	N.D	7.0
35	1.5	7.1	N.D	7.8	N.D	FIN[b]	N.D	5.2	N.D	8.3

[a] Not detected.
[b] Flock Finished.
[c] Positive after enrichment.

Table 2
Campylobacter caecal counts in the flocks (C) and the BC (T) on the farms tested.

Campylobacter count ($\log_{10}$ CFU/g)										
Time (Days)	Farm 1						Farm 2		Farm 3	
	Flock 1		Flock 2		Flock 3		Flock 4		Flock 5	
	T	C	T	C	T	C	T	C	T	C
0	N.T[a]	N.D	N.T	N.D	N.T	N.D	N.T	N.D	N.T	N.D
7	N.T	N.D	N.T	N.D	N.T	N.D	N.T	N.D	N.T	N.D
14	N.T	N.D	N.T	N.D	N.T	N.D	N.T	N.D	N.T	3.6
21	N.D[b]	4.5	N.D	4.4	N.D	2.9	N.D	N.D	N.D	6.7
28	N.D	7.5	N.D	6.8	N.D	6.2	N.D	N.D	N.D	8.3
35	1.2	7.5	N.D	6.5	N.D	FIN[c]	N.D	5.4	N.D	8.4

[a] Not Tested.
[b] Not Detected.
[c] Flock Finished.

Table 3
Campylobacter air counts in the flocks tested.

Campylobacter count (log_{10} CFU/m^3)					
Time (Days)	Farm 1			Farm 2	Farm 3
	Flock 1	Flock 2	Flock 3	Flock 4	Flock 5
0	0	0	0	0	0
7	0	0	0	0	0
14	0	0	0	0	4.3
21	2.8	0.3	0	0	4.0
28	3.4	2.7	0	0	4.0
35	1.9	3.1	0	0	2.2

Table 4
The mean, minimum and maximum weights (Kg) recorded of the test and control birds in the 5 different flocks after 35 days rearing.

Flock	Average slaughter weight recorded		Minimum slaughter weight recorded		Maximum slaughter weight recorded	
	T	C	T	C	T	C
1	1.6[a*]	1.2[b]	1.5	0.9	1.7	1.5
2	1.9[a]	1.5[b]	1.7	1.1	2.1	1.7
3	1.6[a]	1.1[b]	1.2	0.9	1.8	1.2
4	1.7[a]	1.4[b]	1.3	0.9	2.0	1.7
5	1.7[a]	1.3[b]	1.5	1.2	1.9	1.4

*A different letter denote statistical significance at the 5% level ($p < 0.05$).

within the flock (Jacobs-Reitsma, Van de Giessen, Bolder, & Mulder, 1995) and often by farm staff via the ante-room into the surrounding external environment (Battersby, Whyte, & Bolton, 2016). A contaminated tarmac apron, for example, may then serve as a source of infection for subsequent flocks as *Campylobacter* are picked up on boots, clothes, equipment and hands and carried into the broiler house (Newell et al., 2011). Even when boots and clothes are changed and hands are washed, contaminated door handles and other contact surfaces in the ante-room, after these biosecurity measures, may provide *Campylobacter* that is carried into a new flock (Allen et al., 2008; Battersby et al., 2016).

Thus, farm staff and visitors present a major hazard for the introduction of *Campylobacter* into broiler houses (Food Safety Authority of Ireland, 2011; Newell et al., 2011). Overcoming the sporadic application or inadequacy in the design of biosecurity measures therefore requires the removal of the variable factor or human element. This study demonstrated that preventing direct contact between farm staff and broilers protected the birds from *Campylobacter* infection. As reported in other studies (Bull et al., 2006; Torralbo et al., 2014), all of our flocks became infected with *Campylobacter* after approximately 2–3 weeks. However, with the exception of flock 1 (day 35), all the birds in the biosecure cube remained *Campylobacter* free. Indeed, the flock 1 infection of the test birds was traced to a breach of the rules by the farm staff.

Interestingly, despite the air being *Campylobacter* positive in 3 of the flocks, the test birds remained infection free. Broilers generate copious amounts of dust consisting of dried droppings, feather and skin scales which carry bacteria into the air (Ritz, Mitchell, Fairchild, Czarick, & Worley, 2006) including *Campylobacter* (Olsen, Lund, Skov, Christensen, & Hoorfar, 2009; Søndergaard et al., 2014). Thus the air has been considered as a vehicle facilitating the rapid spread of *Campylobacter* within broiler flocks (Vandeplas et al., 2010). However, our research suggests this may not be the case as the minimum infectious dose of this organism in broilers (currently unknown) may not be achieved.

Despite there being no fly screen to prevent ingress of flies into the biosecure cube, the birds remained *Campylobacter* negative until day 35 in flock 1 and in all subsequent test flocks. Flies have been reported as *Campylobacter* vectors into broiler houses (Hald et al., 2007). However, other research has shown that many flies entering broiler houses are not contaminated with *Campylobacter* (O' Mahoney, Buckley, Bolton, Whyte & Fanning, 2011). Regardless, flies are most efficiently kept out of broiler houses by covering ingress points with a fly screen mesh and it is anticipated that broiler houses using the biosecure cube infrastructure would also have a fly screen veranda outside of the house, protecting the birds.

Our study also demonstrated that the test birds were significantly ($P < 0.05$) heavier than the control broilers at day 35. On average they reached a target weight of 1.2–1.5 kg at least 6 days faster which, assuming 10 days down-time between flocks, would allow up to an extra 2 flocks per annum or an approximate 25% increase in productivity. This was not unexpected as it is generally believed that a higher level of biosecurity contributes to improved health and productivity as demonstrated in pigs, manifested as more efficient feed conversion and weight gain (Laanen et al., 2013; Ribbens et al., 2008). Broilers subject to lower physical and psychological stress, such as those in the biosecure cube, may be less susceptible to infection with bacteria like *Campylobacter* (Humphrey, 2006). Moreover, limiting space for broilers results in lower feed conversion rates, reduced weight, bruising, poor feather development and higher mortalities (Dawkins, Donnelly, & Jones, 2004). It could therefore be argued that although the stocking density was the same inside and outside of the biosecure cube, the birds within the cube were subject to lower stress conditions (due to the smaller flock size and lack of direct contact with the farmer) and this might have contributed to the absence of *Campylobacter* infection. Although research on the effects of flock size on food safety is limited, several studies have investigated the impact of stocking density on welfare and growth. Interestingly these studies report only minor differences in stress (Bolton, Thompson, Jones, & Dewar, 1972; Buijs, Keeling, Rettenbacher, Van Poucke, & Tuyttens, 2009) and behaviour (Febrer, Jones, Donnelly, & Dawkins, 2006; Weeks, Nicol, Sherwin, & Kestin, 1994). Moreover, in contrast to the birds in the biosecurity cube in our study, there were no weight gain differences between low and high density flocks (Buijs et al., 2009). Thus while broiler stress may be influenced by flock size, other factors such as stocking density, humidity and temperature have a bigger impact (Jones, Donnelly, & Stamp Dawkins, 2005).

Although not an objective of this study, more effective biosecurity could also reduce the usage of antimicrobial drugs with all of the positive implications for controlling the spread of resistance amongst bacteria of public health significance (Laanen et al., 2014). Moreover, improved productivity and enhanced profit could help overcome farm staff's scepticism about biosecurity measures (Gunn, Heffernan, Hall, McLeod, & Hovi, 2008; Nöremark, Lindberg, Vågsholm, & Sternberg Lewerin, 2009).

Application of the biosecure cube concept to the entirety of the

broiler house is feasible, not least because similar units are currently in use in commercial pig production. The first unit, used in this study on farm 1, was constructed of durable materials and could be easily dismantled to facilitate cleaning. Full scale application would require walkways to allow farm staff to apply fresh litter when needed, remove dead birds and inspect equipment. Moreover, the biosecure unit in its current form is not suitable for partial depopulation but thinning practices are currently under review.

It was concluded that preventing direct contact between poultry farm staff and broilers considerably enhanced biosecurity resulting in the production of *Campylobacter* free birds. Extending the 'biosecure cube' concept to the entirety of the broiler house would significantly improve food safety, animal welfare and productivity.

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Appendix

Picture 1. Initial BC unit before bird placement.

Picture 2. Secondary BC unit before bird placement.

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