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Sources of *Campylobacter* spp. Colonizing Housed Broiler Flocks during Rearing

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The study aimed to identify sources of campylobacter in 10 housed broiler flocks from three United Kingdom poultry companies. Samples from (i) the breeder flocks, which supplied the broilers, (ii) cleaned and disinfected houses prior to chick placement, (iii) the chickens, and (iv) the environments inside and outside the broiler houses during rearing were examined. Samples were collected at frequent intervals and examined for *Campylobacter* spp. Characterization of the isolates using multilocus sequence typing (MLST), serotyping, phage typing, and *flaA* restriction fragment length polymorphism typing was performed. Seven flocks became colonized during the growing period. *Campylobacter* spp. were detected in the environment surrounding the broiler house, prior to as well as during flock colonization, for six of these flocks. On two occasions, isolates detected in a puddle just prior to the birds being placed were indistinguishable from those colonizing the birds. Once flocks were colonized, indistinguishable strains of campylobacter were found in the feed and water and in the air of the broiler house. *Campylobacter* spp. were also detected in the air up to 30 m downstream of the broiler house, which raises the issue of the role of airborne transmission in the spread of campylobacter. At any time during rearing, broiler flocks were colonized by only one or two types determined by MLST but these changed, with some strains superseding others. In conclusion, the study provided strong evidence for the environment as a source of campylobacters colonizing housed broiler flocks. It also demonstrated colonization by successive campylobacter types determined by MLST during the life of a flock.

Campylobacter spp., especially *C. jejuni* and *C. coli*, are one of the most commonly reported bacterial causes of human enteritis in industrialized countries (World Health Organization [http://www.who.int/mediacentre/factsheets]). In 2003, 44,832 cases were reported in England and Wales (Health Protection Agency [http://www.hpa.org.uk/infections]), and this is a substantial underestimate of the number of cases in the community (1). Chicken meat is frequently contaminated with campylobacter (Food Standards Agency [http://www.food.gov.uk]) (23), and a reduction in the number of poultry products contaminated with campylobacter would bring improvements in public health (38). This measure is endorsed by the United Kingdom Food Standards Agency, which aims to reduce foodborne illness by 20% by 2006 (http://www.food.gov.uk). It is a strongly held view that the main focus for the control of campylobacter in chickens should be on the farm, and it is therefore important to identify the most important sources for flock colonization so that intervention strategies can be developed (2).

There have been many studies of the epidemiology of campylobacter in poultry production, and there is a degree of dispute over which are the most important sources for flock colonization. Vertical transmission from parent flocks (34), carryover from previously positive flocks (35), and horizontal transmission via contaminated water (33), domestic and wild animals (18, 44), and the external environment (27) have all been implicated. Horizontal transmission is generally considered the most significant cause of broiler flock colonization (7, 19), although it has yet to be proven by reproducible observation and strain characterization (29). Many studies have not detected campylobacter in the environment until after the flock is colonized, and the direction of the spread is thus unclear. Likewise, limited sampling and strain characterization have, in some cases, hindered the identification of sources.

This project aimed to undertake detailed sampling of as many potential points of entry of the target pathogen as possible and used several typing methods to establish the relationships between these isolates and those found in the broiler flocks. Investigations were performed between 2000 and 2001 at three United Kingdom poultry companies. Ten broiler flocks and their environments inside and outside the poultry house, including air, were sampled frequently and cultured for

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the presence of *Campylobacter*. The parent flocks were also sampled. Speciation, serotyping, phage typing, multilocus sequence typing (MLST), and *flaA* restriction fragment length polymorphism were used to characterize the isolates.

MATERIALS AND METHODS

Isolation and identification of campylobacter. The samples collected were examined by enrichment culture to determine presence/absence of campylobacter and by direct plating to determine numbers of campylobacter. Enrichment was performed in modified Exeter broth (MEB), consisting of Bolton broth (27.6 g liter^{-1}, CM983; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), *Campylobacter* growth supplement (sodium metabisulphate, sodium pyruvate, and ferrous sulfate, all at 250 mg liter^{-1}, SV61; Mast Diagnostics, Mast Group Ltd., Bootle, United Kingdom), *Campylobacter* selective supplement (trimethoprim, 10 mg liter^{-1}; rifampin, 5 mg liter^{-1}; polymyxin B, 2,500 IU liter^{-1}; cefoperazone, 15 mg liter^{-1}; and amphotericin B, 2 mg liter^{-1}; SV59 [Mast]), and lysed defibrinated horse blood (10 ml liter^{-1}; E&O Laboratories, Bonnybridge, Scotland). Samples were incubated in containers with a small headspace and tightly closed lids at 37°C for 48 h and 96 h. After enrichment, 10 μl of the enrichment broth was streaked onto charcoal cefoperazone deoxycholate agar (CCDA) (CM739 with SR155 supplement; Oxoid) and the plates were incubated at 37°C for 48 h in a microaerobic atmosphere. This was achieved by evacuating the air from gas jars (Don Whitley Scientific Ltd., West Yorkshire, United Kingdom, and Launch Diagnostics Ltd., Kent, United Kingdom) and replacing it with a gas mixture that resulted in an atmosphere comprising 5 to 6% O_2, 3 to 7% CO_2, and 7% H_2 in a balance of nitrogen. Some samples were plated directly onto CCDA and incubated as described above. Between three and six campylobacter colonies per positive sample were confirmed by light microscopy for typical spiral-shaped cells and rapid motility, a positive oxidase test, and lack of growth in air (at 20 to 25°C for 48 h). Isolates were stored at −80°C in cryovials (Pro-Lab Diagnostics, Neston, Cheshire, United Kingdom) until subtype analysis could be performed.

Between one and six confirmed isolates from most positive samples were characterized. Speciation using real-time PCR (5), serotyping by the heat-stable antigen scheme of Frost et al. (11), and phage typing (12) was performed at the *Campylobacter* Reference Unit, Health Protection Agency, Colindale, London, United Kingdom. In the serotyping scheme, strains were labeled according to which antiserum gave the strongest reaction, as many isolates cross-react with several antisera. Subtypes were defined by the combined results of phage typing and serotyping. *Campylobacter* strains were also characterized by MLST (8). *fla* typing was performed using the protocol recommended by the CAMPYNET research forum (http://campynet.vetinst.dk/Fla.htm), which amplifies the full *flaA* gene and digests it using DdeI. The protocol was modified so that digestion was performed using 1 μl of sterile ultrapure water, 2 μl of 10× enzyme buffer, 1 μl of 5 U DdeI (New England Life Sciences, Hitchin, United Kingdom), and 16 μl of flagellin PCR product. Other reaction conditions were as described above.

Sampling. Ten housed broiler flocks (flocks A to J) were studied over their 5- to 7-week life spans. Flocks A and B, supplied by company 1, were grown on one farm and flocks C, D, and E, supplied by company 2, on another. The poultry companies selected the farms for the study. Flocks A and B were reared in adjacent houses over the same growing period, as were flocks C and D. These flocks were partially depopulated in the sixth week of the growing cycle, at which point sampling was discontinued. In contrast, flocks F to J were reared on four farms from company 3 that were selected by the research team and were not partially depopulated. Flocks G and H were reared on the same farm but at different times of year. Studies were also performed on three additional flocks, X to Z, in the final week of life, primarily to test the air inside and outside the broiler sheds. Flock X was reared on the same farm as flocks G and H but at a different time of year, and flocks Y and Z were reared on different farms. This study was not designed to evaluate seasonal differences in flock colonization.

Samples were collected at least every 7 days and sometimes more frequently from (i) breeder flocks, which supplied the broilers, (ii) the cleaned and disinfected broiler houses prior to chick placement, (iii) the chickens, and (iv) the environments inside and outside the broiler houses. Samples were transported to the laboratory in a cool box and processed on the same day as collection.

Breeder flocks. Fecal samples were collected from the floor of every house of each breeder farm supplying fertile hatching eggs for the broiler flocks under investigation. Whenever possible, these were collected 21 days prior to chick placement. On average, 28 samples from breeders supplying each broiler flock were examined (breeders associated with flock E were not tested, due to technical difficulties). The samples were tested by enrichment culture and direct plating.

Broilers. Two to 14 composite samples from day-old chicks were tested by enriching 5 g of lining paper soiled with feces, from crates in which 125 chicks were transported, in 225 ml of MEB. Chickens were then sampled at least weekly by collecting between 10 and 14 individual fresh feces or cloacal droppings from the broiler house floor. If feces could not be found (an occasional occurrence in the first 2 weeks of the flock's life), samples were obtained from the cloacae of individual birds by using Pernasal swabs (Medical Wire and Equipment, Corsham, Wiltshire, United Kingdom). Ten to 14 ceca were collected from the abattoir on the day of kill. Fecal and cecal samples were streaked directly onto CCDA, and 2 g of sample was enriched with 18 ml of MEB (for flocks A to E and X to Z) or 1 g of sample with 9 ml of MEB (for flocks F to J). Swabs were enriched with 9 ml of MEB. If campylobacters in samples were enumerated, appropriate dilutions were made using maximum recovery diluent (CM 733; Oxoid) and spread onto duplicate CCDA plates. After incubation, several colonies were checked by microscopy and those with typical morphology were counted. The number of presumptive campylobacters per g of fecal material was calculated.

Environments inside and outside the broiler house. Two composite samples (from three to six areas of the broiler house) of litter and feed from the feeders and one composite sample of water from the drinkers were collected at each sampling visit throughout the rearing period. Twenty-five grams of food or litter was enriched in 225 ml of MEB, and 125 ml of water was enriched in the equivalent volume of double-strength MEB.

Three methods were used to sample air inside the broiler house. For flocks A to J, four Columbia blood agar (CBA) plates (CM331 [Oxoid] with 5% defibrinated horse blood [E&O Laboratories]) per sampling visit were placed on stands 50 cm above the ground and exposed to settling particulate matter (>10 μm in diameter) for 5 min. At the same time, two Burkard single agar plate samplers (Burkard Manufacturing Co. Ltd., Rickmansworth, United Kingdom) operating at a flow rate of 20 liters min^{-1} were used to collect samples of aerosols and particulate matter <10 μm in diameter onto CBA plates. The samplers were placed on stands 25 cm above the ground that sheltered them from settling particulate matter. For flocks X to Z, 35 samples of settling particulate matter and six aerosol samples (collected using Burkard single agar plate samplers) were collected from one sampling visit made in the last week of the bird's life. The contents of the CBA plates were subsequently enriched with 225 ml of MEB. For flocks X to Z, four additional samples were collected using a stainless steel cyclone sampler made at the Silsoe Research Institute. This was mounted on either a stand or a telescopic mast in order to sample air from different areas of the broiler house. The sampler was operated at a flow rate of 750 liters min^{-1} for 10 to 15 min. Particulate matter was captured into 80 ml of MEB, which was injected by peristaltic pump into the cyclone sampler, at a flow rate of 10 ml min^{-1} and recirculated from the reservoir at its base. Six samples of air exiting each broiler house, on some occasions up to 30 m downwind, were also collected using the cyclone sampler mounted on a telescopic mast. The contents of the MEB were enriched.

Many items inside and outside the broiler growing area were sampled opportunistically. The numbers of samples collected at particular times are shown in Table 1.

Samples from walls, floors, structural supports, feed dispensers, the anteroom floors and doors, concrete aprons and paths, stockman's boots, and transport crates were obtained by rubbing a cotton wool swab moistened with maximum recovery diluent over ~0.1 m^2 of the object's surface. Samples from drinkers, fans, heaters, and weighing machines were obtained by swabbing four pieces of the same equipment and pooling the swabs. Swabs were enriched in 225 ml of MEB.

Straw, mud, feces from other animals (cow, horse, sheep, dog, fox, hedgehog, rabbit, mouse, shrew, and wild bird), and crushed litter beetles were enriched with MEB using a 1:10 ratio of sample to broth.

Ten milliliters of water from puddles was enriched with an equivalent volume of double-strength MEB for flocks A to E, and 25 ml was enriched with 225 ml of MEB for flocks F to J. Occasionally, campylobacters in puddle samples were enumerated using a nine-tube most-probable-number test (37) to estimate the number of campylobacters per ml of water.

RESULTS

Seven of the 10 broiler flocks became colonized with *Campylobacter* spp. during the growing period (flocks A, B, F, G, H, I, and J). Campylobacter was first detected in one of these

TABLE 1. Sources of *Campylobacter* spp. recovered from flocks and the environment

Flock(s)	Sample type[c]	No. of positive samples/no. of samples tested[a] for birds of indicated ages (days):						
		0–1*	6–16**	18–28**	25–28**	30–33	35–40**	42–49***
A/B[b]	Broilers	0/2[e]	0/10	0/10	**1/10**; 0/10[d]	—	**10/10**	—
	Puddles	**1/1**[e]	0/8	0/4	0/4	—	0/4	—
	Other	0/10[d, e, su, h, sh]	0/12[d, e, su, w, sh]	0/6[d, e, su, w, sh]	0/6[d, e, su, w, sh]	—	**10/16**[d, e, su, w, sh, **cr**]	—
C/D[b]	Broilers	0/2[e]	0/22	0/11	0/11	—	0/11	0/11
	Puddles	0/2	**4/7**	0/4	0/2	—	0/2	0/2
	Other	0/8[d, e, su, do, w]	0/16[d, e, su, do, w]	0/8[d, e, su, do, w]	0/8[d, e, su, do, w]	—	0/8[d, e, su, do, w]	0/18[d, e, su, do, w, cr]
E	Broilers	0/2[e]	0/22	0/11	0/11	—	0/11	0/11[t]
	Puddles	0/4	0/7	0/3	0/3	—	**3/3**	0/3
	Other	0/8[d, e, su, do, w]	0/8[d, su, do, w]	0/4[d, do, w]	0/4[d, su, do, w]	—	0/4[d, su, do, w]	0/9[d, su, do, w, cr]
F	Broilers	0/12[e]	0/20	0/11	—	0/10	0/10	**3/10**
	Puddles	0/6	**1/6**	0/2	—	0/4	0/2	—
	Shed surround	0/5	0/6	0/1	—	0/2	0/1	—
	Other	0/8[su, f, st]	0/3[st, i]					**3/5**[cr]
G	Broilers	0/10[e]	0/21	0/20	0/10	**10/10**	**5/5**	**10/10**
	Puddles	**1/2**	0/2	—	—	0/1	—	—
	Shed surround	0/1	0/1	0/3	0/2	0/2	—	—
	Other	0/9[su, e, w]	0/1[e]	0/1[w]	**1/5**[c, d, st]	**1/1**[c]	—	**3/5**[cr]
H	Broilers	0/14[e]	0/28	**1/28**	**14/14**	**14/14**	**28/28**	**14/14**
	Puddles	**2/3**	**2/4**	**1/2**	—	**1/1**	**1/1**	—
	Shed surround	0/4	**1/4**[pa]	**1/2**[pa]	—	—	—	—
	Other	**1/10**[c, w, su, d, e]	0/1[c]	—	—	—	—	**5/5**[cr]
I	Broilers	0/14[e]	0/28	0/14	—	0/14	0/14	**2/59**
	Puddles	—	0/2	0/1	—	—	—	—
	Shed surround	0/2	0/3	0/2	—	0/1	0/1	—
	Other	0/11[w, su, d, i]	0/10[d, su, e]	0/2[i, d]	—	0/3[d]	—	—
J	Broilers	0/14[e]	0/28	0/28	0/28	**1/14**	**14/14**	**13/13**
	Puddles	0/2	0/5	**2/3**	**1/4**	0/2	0/1	—
	Shed surround	0/3	0/2	—	0/1	—	—	—
	Other	0/12[c, d, su]	0/10[d, c, su]	0/4[w, d]	0/5[d, su]	0/3[d]	**1/4**[d, su]	**5/5**[cr]

[a] Bold type indicates positive results. *, prefill to chick placement; **, may represent more than one sampling visit; ***, day of kill; —, not sampled; c, cow feces; cr, transport crates (pre-bird loading); d, drinker surfaces; do, dog feces; e, equipment; f, floor of anteroom or shed; h, horse feces; i, insects; pa, path; sh, sheep feces; st, straw outside shed; su, structural supports inside shed, and walls; w, wild animals, including bird, fox, hedgehog, mouse, rabbit, and shrew.

[b] Flocks were grown concurrently.

[c] Samples from broiler flocks were obtained from fecal, cloacal, or cecal material. Shed surround includes concrete aprons and paths.

[d] First result is from flock A, and second result is from flock B.

[e] Number of composite samples tested.

flocks after 18 days, in four flocks between 28 and 33 days, and at depletion for two other flocks (Table 1). High levels of campylobacter were detected in fecal samples within 1 week of this organism first being detected in the flocks ($\log_{10}$ campylobacter per g of feces [means ± standard errors] were 5.4 ± 0.3, 5.1 ± 0.2, 6.1 ± 0.4, and 4.6 ± 0.4 for flocks A, B, H, and J, respectively). The level of campylobacter did not change significantly as the flock continued to grow, e.g., flock H had $\log_{10}$ campylobacter per g of feces of 6.1 ± 0.4 on day 28, 6.2 ± 0.5 on day 30, and 6.7 ± 0.3 on day 35 (with a P value of 0.48, using a one-way analysis of variance test).

Five broiler flocks were colonized exclusively with *C. jejuni* (flocks A, B, G, I, and J), another exclusively with *C. coli* (flock F), and one (flock H) with both species (Table 2). Further characterization of isolates from the flocks that were campylobacter positive before the day of depletion showed that flocks A, B, and J were colonized by only one sequence type (ST), as determined by MLST, while flocks G and H were colonized by at least two types that were genetically unrelated (i.e., did not belong to the same clonal complex). In these flocks, the type of campylobacter that first colonized the birds was gradually superseded and sometimes replaced by other types (Table 2). For example, flock G was initially colonized by *Campylobacter jejuni* phage type 2 but the dominance of this type was significantly reduced during the rearing period (P value of 0.026 between days 33 and 40 and <0.001 between days 40 and 45, using the chi-square exact test). At the end of the flock's life, phage type 67 was the most dominant subtype. The first isolates detected in flock H were strains of *C. coli*, but these were rapidly replaced by *C. jejuni* ST 791. This ST dominated until day 28 but was found in significantly lower proportions from this point on (P values of <0.001, <0.001, and 0.015 between day 28 and days 30, 35, and 44, respectively). At slaughter, two subtypes of *C. jejuni* (ST 791 and ST 354) and two subtypes of *C. coli* (different from those originally colonizing the flock) were detected. Occasionally, more than one ST was detected in

TABLE 2. *Campylobacter* species and types detected in relation to sample type and collection time[d]

Flock(s)	Sample source(s) (day[s])	Species (*n*)	ST[a] (*n*)	Phage type/serotype[b] (*n*)	*flaA*[c] (*n*)
A/B	Breeders (0) from one farm with six houses	*C. jejuni* (9)	**447** (2)	**1**/13 (1); **33**/13 (1)	—
			436 (2)	**1**/NT (2)	—
			311 (1)	**63**/13 (1)	—
			1012 (1)	**33**/13 (1)	—
			—	**63**/13 (2); **1**/13 (1)	
		C. coli (10)	—	—	—
A	Broilers (35)	*C. jejuni* (26)	**447** (8)	**33**/13 (2), 7 (2), 57 (1); **1**/NT (2), 7 (1)	—
			—	**33**/13 (9), 7 (3), NT (1); **1**/NT (4), 13 (1)	—
B	Broilers (35)	*C. jejuni* (18)	**447** (10)	**33**/13 (2), 7 (2); **1**/13 (2), NT (1), 2 (1), 7 (1), 21 (1)	—
			—	**33**/13 (7), 7 (2), NT (2), 57 (1); **1**/NT (5), 5 (1)	—
A/B	Puddle (0)	*C. jejuni* (3)	**436** (3)	**1**/NT (2), 18 (1)	—
F	Breeders (21) from four farms and 13 houses	*C. jejuni* (61)	—	—	—
	Broilers (43)	*C. coli* (12)	860 (10)	**44**/56 (7), NT (3)	—
			887 (1)	**44**/24 (1)	—
			— (1)	**1**/48 (1)	—
	Puddle (0)	*C. jejuni* (1)	—	**1**/6 (1)	—
	Transport crates (43)	*C. coli* (13)	1055 (4)	**7**/NT (2); **NT**/NT (2)	—
			855 (3)	**44**/56 (3)	—
			860 (2)	**NT**/NT (2)	—
			887 (1)	**2**/24 (1)	—
			1597 (3)	**44**/24 (3)	—
		C. jejuni (1)	— (1)	**33**/5 (1)	—
G	Breeders (21) from four farms and 11 houses	*C. coli* (21)	—	—	—
		C. jejuni (94)	**262** (3)	**14**/50 (2); **33**/50 (1)	—
			104 (5)	**34**/50 (3), 34 (1); **5**/50 (1)	—
			137 (4)	**8**/NT (1), 37 (1), 55 (1); **1**/NT (1)	—
			93 (3)	**RDNC**/50 (3)	—
			574 (2)	**1**/50 (2)	—
			21 (1)	**33**/19 (1)	—
			806 (3)	**14**/50 (3)	—
			1015 (1)	**14**/50 (1)	—
			—	**14**/50 (17), NT (1); **8**/NT (10), 37 (2); **RDNC**/50 (10); **1**/NT (4), 50 (4), 55 (1); **33**/NT (3), 19 (3), 50 (2); **NT**/55 (2); **25**/50 (1); **34**/50 (11); **39**/31 (1)	—
	Broilers (33)	*C. jejuni* (31)	**354** (14)	**2**/18 (14)	**F2** (1)
			—	**2**/18 (17)	—
	Broilers (40)	*C. jejuni* (14)	**354** (1)	**2**/18 (1)	—
			45 (1)	**67**/27 (1)	**F1** (1)
			—	**2**/18 (10)	—
			—	**67**/45 (2)	**F1** (2)
	Broilers (45)	*C. jejuni* (26)	**45** (22)	**67**/27 (9), 45 (4), 13 (2), NT (1); **RDNC**/45 (4), 13 (2)	**F1** (5)
			354 (3)	**2**/18 (3)	**F2** (1)
			—	**67**/45 (1)	—
	Puddle (0)	*C. jejuni* (1)	**45** (1)	**67**/27 (1)	**F1** (1)
	Cattle (126–33)	*C. coli* (2)	—	**NT**/NT (2)	—
		C. jejuni (2)	**21** (2)	**33**/1 (1), 19 (1)	—
	Anteroom (26)	*C. jejuni* (3)	**677** (3)	**1**/NT (3)	—
	Feed, air, and water (33–40)	*C. jejuni* (13)	—	**2**/18 (13)	—
H	Breeders (21) from three farms and 11 houses	*C. jejuni* (17)	**50** (4)	**14**/NT (1)	—
			267 (3)	**1**/37 (1)	—
			21 (3)	**NT**/NT (1)	—
			45 (3)	**2**/NT (1)	—

Continued on following page

TABLE 2—*Continued*

Flock(s)	Sample source(s) (day[s])	Species (*n*)	ST[a] (*n*)	Phage type/serotype[b] (*n*)	*flaA*[c] (*n*)
			791 (2)	**RDNC**/NT (2)	**F6** (2)
			324 (1)	—	—
			353 (1)	—	—
		C. coli (3)	—	**7**/66 (1); **2**/66 (1); **44**/NT (1)	—
	Broilers (18)	*C. coli* (3)	—	**NT**/61 (2), 28 (1)	—
	Litter (21)	*C. jejuni* (2)	**791** (2)	**1**/9 (1); **NT**/9 (1)	**F5** (1)
	Broilers (28)	*C. jejuni* (10)	**791** (10)	**NT**/9 (7); **1**/9 (3)	**F5** (3)
	Broilers (30)	*C. jejuni* (13)	**354** (10)	**2**/18 (2)	—
			791 (3)	**44**/9 (1); **NT**/NT (1)	**F5** (1)
	Broilers (35)	*C. jejuni* (14)	354 (13)	**2**/18 (3)	**F2** (3)
			791 (1)	**44**/9 (1)	—
	Broilers (44)	*C. jejuni* (7)	**791** (6)	—	—
			354 (1)	—	—
		C. coli (5)	—	**2**/39 (4); **44**/NT (1)	—
	Puddles (0–21)	*C. jejuni* (15)	**42** (9)	**1**/13 (4); **33**/9 (1)	—
			257 (3)	—	—
			354 (1)	**2**/18 (1)	**F2** (1)
			262 (1)	—	—
			790 (1)	—	—
	Cattle (0)	*C. hyointestinalis* (3)	—	—	—
	Path (15)	*C. jejuni* (3)	**42** (3)	**1**/13 (1)	—
	Air (30–35)	*C. jejuni* (5)	**354** (5)	—	—
J	Breeders (21) from one farm and three houses	*C. jejuni* (109)	**50** (17)	**2**/13 (6), 44 (3), NT (2), 60 (2), 18 (1); **NT**/13 (1); **44**/13 (1); **1**/3 (1)	**F7** (1)
			257 (2)	**2**/63 (1); **NT**/13 (1)	—
			—	**2**/13 (57), NT (19), 60 (11); **NT**/63 (1), 13 (1)	—
	Puddle (18–25)	*C. coli* (5)	—	**2**/NT (4), 39 (1)	—
	Broiler (32)	*C. jejun*i (3)	**573** (1)	**1**/3 (1)	**F5** (1)
			—	**1**/3 (2)	—
	Broiler (39)	*C. jejuni* (42)	**573** (6)	**1**/3 (3), 13(2), NT (1)	—
			—	**1**/3 (25), NT (11)	—
	Drinker (39)	*C. jejuni* (3)	**573** (1)	**1**/3 (1)	—
			—	**1**/3 (2)	—
	Broiler (42)	*C. jejuni* (38)	573 (3)	**1**/3 (3)	—
			—	**1**/3 (28), NT (7)	—
X	Broiler (38)	*C. coli* (12)	—	**2**/48 (9), 24 (1), NT (2)	—
	Air in shed (38)	*C. coli* (2)	—	**2**/48 (2)	—
		C. jejuni (1)	—	**6**/NT (1)	—

[a] Determined by MLST. Bold type indicates sequence type.
[b] When serotype is preceded by a comma, isolates have the same phage type (in bold) as the previous set of isolates. NT, nontypeable; RDNC, reacted with phage but did not conform to a recognized type.
[c] *flaA* restriction fragment length polymorphism type. Band sizes, measured manually, are as follows (mean numbers of base pairs ± standard errors): for F1, 177 ± 3, 234 ± 1, 258 ± 1, 329 ± 2, and 610 ± 4; for F2, 161 ± 3, 232 ± 1, and 938 ± 7; for F5, 161 ± 1, 224 ± 2, 281 ± 3, and 896 ± 4; for F6, 159, 187, 223, 271, and 410; and for F7, 162, 190, 229, 302, and 333.
[d] —, not typed.

individual birds (data not shown). A greater diversity of types was detected using phage typing/serotyping. For example, flocks A, B, and G were colonized with between four and eight phage types/serotypes.

Eighty-three percent (189/229) of the fecal samples collected from the breeder flocks were campylobacter positive. All broiler flocks (apart from flock E, which was not tested) contained some chicks that had hatched from eggs laid by campylobacter-positive hens. The types of campylobacter isolated from the flocks that supplied chicks to flocks F, G, H, and J were different from those isolated from the broilers, although one of the types isolated from the breeders which supplied flock H had the same ST but a different phage type, serotype, and *fla* type from that isolated from the broiler flock (Table 2). The types of campylobacter detected in the parents of flocks A and B were indistinguishable, however, from those first detected in the broilers on day 35.

Campylobacter was isolated from the environment surrounding the broiler house of six of the seven colonized flocks but also of three negative flocks (Table 1). In all cases, the environment was positive prior to as well as during flock colonization but only a small proportion of the environmental samples examined were positive (27/395, ~7%), with campylobacter being isolated most frequently from puddles (20/119, ~16%).

The campylobacter isolates from the six flocks where the environment was positive prior to flock colonization were characterized in more detail. In flocks G and H (which were reared

in the same broiler house 6 months apart), two different strains found in a puddle just prior to the birds being placed were indistinguishable by MLST, phage typing, serotyping, and *flaA* typing from those later identified in the flocks towards the end of their lives until slaughter (Table 2). The puddles from which the strains were isolated were positioned on a path leading between the broiler house, a milking parlor, and two other broiler houses approximately 75 m away. Enumeration of campylobacters in this puddle on days 21, 30, and 35, while flock H was colonized, detected 2.4, >18, and 0.22 CFU of *Campylobacter* spp. per ml of water, respectively. When the criteria for genetic relatedness were reduced to MLST clonal complex (isolates sharing a minimum of four identical alleles), no further linkages between campylobacters in the environment and those colonizing broiler flocks were found.

Campylobacter was not detected in the litter, feed, water, or air of the broiler houses (n = 116, 103, 68, and 338 samples, respectively) while the flocks were campylobacter negative. Once the flocks were positive, however, it was found in 3 of 18 litter samples (flocks G, H, and J), 1 of 19 feed samples (flock G), 4 of 13 water samples (flock G), and 15 of 248 air samples (flocks $G^{Burkard}$, $H^{Burkard}$, $X^{Burkard}$, X^{settle}, $Y^{Burkard}$, $Y^{cyclone}$, and $Z^{cyclone}$ [the superscript text indicates the sampling method]) collected from inside the house. Campylobacter was also detected on four occasions (n = 18) in the air up to 30 m downwind of the broiler house (flocks X and Y). Whenever isolates from inside the house or the air outside the house were characterized, they were indistinguishable from the dominant subtype of campylobacter in the flock at the time (Table 2). The anterooms of the broiler houses were also sampled prior to flock colonization. Campylobacter was detected in 1 of 142 samples (flock G), but the isolate was genetically distinct from the strains that colonized the broiler flock.

Campylobacter was detected on 26 of 45 (58%) transport crates to be used to take birds for slaughter, on arrival of the crates at the farm (Table 1). Isolates from crates used to transport flock F, which was campylobacter negative a few days prior to depopulation but positive at the abattoir, were characterized. These isolates were indistinguishable from some of those found in the flock (Table 2). Birds had been held in the transport crates for over 6 hours.

DISCUSSION

Poultry meat is considered to be an important source of zoonotic campylobacter infections in developed countries, and it is hoped that reduction in the contamination of poultry will bring improvements in public health. There is therefore a need to improve our understanding of the epidemiology of campylobacter in poultry, in order to formulate control measures which can prevent flock colonization. This study, in common with others (18, 22), found that campylobacter was rarely isolated from housed broiler flocks until the birds were at least 3 weeks old. There is currently no agreement on the reasons for the delay in colonization, but it is unlikely to be due to lack of exposure to *Campylobacter*. Maternal antibodies in young chicks have been suggested to have a role (39). Strategies that may exclude the bacterium from the flock for a further 3 to 5 weeks, until slaughter age, include the use of sustained and effective biosecurity or the development of competitive exclusion agents that would improve the resistance of birds to colonization with campylobacter (2). Identification of the sources of flock colonization would enable biosecurity measures to be targeted towards the areas posing the greatest risk.

Multiple sequence types of campylobacter, as determined by MLST, were detected in two of the five broiler flocks that were positive before the day of depletion. This is in accordance with data presented by Shreeve et al. (41) and Hein et al. (16), who found that 40 and 77% of flocks, respectively, were colonized with more than one campylobacter genotype. Individual birds were also sometimes colonized with more than one ST. This was first recorded by Schouls and colleagues (40) after examining isolates from three laying hens. In this study, the campylobacter type that first colonized the birds was gradually superseded and sometimes replaced entirely by other types. This could reflect frequent ingress of campylobacters perhaps with differing colonization potentials (36). It also highlights the difficulties that may be encountered when trying to prevent bird colonization by using live campylobacter vaccines (3). These results also emphasize the need to characterize multiple isolates from a sufficient number of samples in order to understand the complex nature of broiler flock colonization.

The method used to characterize isolates will also influence the interpretation of the epidemiological data obtained. In this study, sequence types determined by MLST frequently comprised several phage types/serotypes. In some cases, it was questionable whether isolates with different serotypes but identical phage types, STs, and *flaA* types were truly different strains. These isolates gave a strong reaction with several antisera from the serotyping scheme and could possibly be phase variants of each other (data not shown). Other workers have recorded similar concerns and the problems that may be encountered when interpreting serotyping data (9, 31). Of the three typing methods used in this study, MLST provided a sound basis for answering epidemiological questions, while the other methods confirmed findings and in some cases further discriminated between the strains. If MLST had been the only method used in this study, we would have drawn the same conclusions about vertical and horizontal transmission on all but one occasion, where a parent flock isolate, ST 791, would have appeared indistinguishable from those colonizing the broiler flock (flock H). If only phage typing/serotyping were used, our conclusions may have been different on more than one occasion (flocks A/B and J), partly as a result of the ambiguity created when isolates were nontypeable by one or both of the methods. Using only one typing method may have led to false conclusions being drawn.

There is a continuing debate about the relative contribution of vertical transmission of campylobacter from parent flocks to their offspring. If vertical transmission occurred, colonization with identical subtypes would be expected. We found that, despite the colonization of some birds in all of the parent flocks, 3 of 10 broiler flocks were not colonized during their lives. Different subtypes of *Campylobacter* were also isolated from the parents and progeny for four of the six positive flocks from which isolates were characterized. For the remaining two concurrently reared flocks, some of the isolates from the parents were indistinguishable from those which were most prevalent in the colonized broilers. The breeder and broiler farms, however, were less than 0.5 mile apart, and it is also possible

that vehicles visiting both farms may have transferred campylobacter from the breeders to the broilers by means other than vertical transmission. This is supported by the late colonization of the two broiler flocks. Thus, overall we consider that vertical transmission was not a major source of colonization for the flocks in this study.

Campylobacter was found in the environment surrounding the broiler house on all but one occasion and more frequently than in other studies, especially prior to flock colonization (14, 18, 43). Improved isolation may have resulted from the increased level of sampling applied in this study (395 samples were tested from the environment) and the enrichment culture method used, which may be better able to recover damaged cells. Even so, *Campylobacter* was detected in only 7% of environmental samples. Puddles were most frequently contaminated, possibly due to protection from desiccation, a stress to which campylobacters are particularly sensitive (20, 30).

The subtypes of campylobacter isolated from puddles just prior to chick placement were indistinguishable from isolates found in two broiler flocks towards the end of their lives. Newell (27) and Hiett et al. (18) also found isolates in a puddle indistinguishable from those in a flock, prior to the colonization, for 1 of 4 and 1 of 16 broiler flocks, respectively. Under normal circumstances, it is likely that only low numbers of campylobacter from the environment will enter the broiler house, and if these are stressed due to conditions not conducive for growth (e.g., exposure to harmful compounds, air, or low levels of nutrients) their colonization potential may be compromised (26). This may have resulted in the delay between detection of certain subtypes of campylobacter in the puddles and detection in the broiler flocks. Once the first birds were colonized, however, these subtypes appeared to spread rapidly, and this may reflect strains regaining improved colonization efficacy after passage through a host (6). Puddles were located on paths, but their source of contamination was unknown. Previously positive flocks reared on the farm might have contaminated them, and this may be a mechanism by which flock-to-flock carryover occurs.

The birds in the flocks were always colonized with some strains of campylobacter that were not detected in any of the other samples related to the flock, i.e., collected from the environment or other animals. Feces from dogs, sheep, horses, wild birds, and mammals and swabs taken from the cleaned and disinfected broiler houses and equipment were all campylobacter negative. Cattle reared on the farms were positive but, in contrast to results from several other studies, harbored subtypes different from those isolated from the flocks (15, 25, 44). While links between these samples and the birds were not found, this does not necessarily mean that links are not there. Increasing the number of samples collected and the number of isolates characterized may further improve source detection, although this study did address this issue more thoroughly than previously published research. In addition, some strains of campylobacter may not have been isolated from samples which were positive only by enrichment culture, as one study suggested that this method has been shown to preferentially select some strains of campylobacter over others (28).

In this study, most birds were colonized with campylobacter within a week of the organism first being detected in the flock. Soon after, indistinguishable strains of campylobacter were found in the feed, water, and air and on drinkers in the broiler house, which may be some of the ways by which campylobacter spreads through a flock. While this point has been raised by other workers (4, 10, 14, 17), it has not until now been confirmed by isolate characterization. Transmission of campylobacter via the air may also be important for spreading the organism between broiler flocks, as campylobacter was detected in the air exiting broiler sheds. This would be especially pertinent if lower doses of campylobacter, as with salmonella (24), were able to cause an infection when given to chickens as an aerosol rather than orally.

The three broiler flocks that were not colonized with campylobacter at slaughter were all reared on the same farm. It may be that good biosecurity was practiced on this farm, as some environmental samples collected from this farm while these flocks were reared were positive for campylobacters. Studies have shown that when farm staff dip their footwear in a disinfectant that is replenished frequently or change into dedicated sets of clothing and footwear which are located behind hygiene barriers, it is possible to either prevent or delay flock colonization (13, 21, 32, 41). This particular farm also had a large area of concrete surrounding the broiler houses. Measures such as introducing wider concrete aprons may be able to reduce the transfer of campylobacter from environmental sources to broiler flocks by increasing the buffer zone and has been recommended by the ACMSF (2). In light of this study, it may also be beneficial to improve the construction, drainage, and maintenance of pathways, as this may reduce the areas in which puddles can form. Although there is debate about the practicalities of maintaining rigorous biosecurity long-term and the cost implications, there is a general consensus within the scientific community that the number of positive flocks can be reduced by these methods (2).

It is possible that the crates used to transport birds to the abattoir could have been the source of contamination for two flocks that were negative on the farm but partly colonized with campylobacter at slaughter (flocks F and I). This study and others (28, 42) found that campylobacter was frequently isolated from transport crates (prior to bird loading), and some of the subtypes isolated from flock F were indistinguishable from those found in the crates used to transport the birds. Herman and colleagues (17) also found evidence to suggest that colonization of broiler flocks occurred during transport. We should exercise caution, however, as we may find that the campylobacter subtypes isolated are common colonizers of broiler flocks.

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