APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2007, p. 5125–5129 Vol. 73, No. 16
0099-2240/07/$08.00+0 doi:10.1128/AEM.00884-07

Use of Multilocus Sequence Typing To Investigate the Association between the Presence of *Campylobacter* spp. in Broiler Drinking Water and *Campylobacter* Colonization in Broilers[▽]

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Received 19 April 2007/Accepted 18 June 2007

The presence of campylobacters in broiler chickens and throughout the broiler water delivery systems of 12 farms in northeastern Scotland was investigated by sensitive enrichment methods and large-volume filtration. *Campylobacter* presence was independent of the water source and whether the water was treated. The genotypes of *Campylobacter jejuni* isolates recovered from chickens and various locations within the water delivery systems were compared by multilocus sequence typing. Matching strains in shed header tanks and birds were found at 1 of the 12 farms investigated. However, the sequence of contamination or whether the source was within or outside the shed was not determined. Nevertheless, these data provide evidence that drinking water could be associated with broiler infection by campylobacters.

Laboratory notifications show *Campylobacter* spp. to be the major cause of identified bacterial human gastrointestinal disease in the United Kingdom. In 2006, the number of cases reached 4,857 in Scotland (95.3 per 100,000; http://www.hps.scot.nhs.uk/giz/wrdetail.aspx?id=32791&wrtype=6). In England and Wales, the 2006 number was 45,290 (87.0 per 100,000; http://www.hpa.org.uk/infections/topics_az/campy/data_ew.htm). Importantly, the Infectious Intestinal Disease Study in England (34) estimated that for every reported case, 7.6 cases occurred in the community, indicating that the real incidence of disease is significantly higher than that reported.

The source of *Campylobacter* infections is not fully understood, although it has been linked to the consumption of poultry meat (28). A recent survey in the United Kingdom has shown that a high proportion (89%) of fresh retail poultry meat in Scotland was contaminated with campylobacters (Food Standards Agency 2001; www.food.gov.uk). The proportion of poultry flocks colonized with campylobacters at slaughter in the United Kingdom is unknown, but limited cross-sectional surveys indicate that this can be as high as 95%, depending on the season (24). A number of studies have addressed biosecurity measures in the broiler house, where infection from a number of environmental sources is suspected (1, 9, 23, 24). Campylobacters cannot normally grow outside the host gut, but they colonize many domestic and wild mammals and birds and survive for various periods in the farming environment. Multiple potential routes of transmission into the broiler house exist, and epidemiological studies indicate these include farm staff (15), insects (13), rodents (14), and aerosols (29). Broiler house drinking water has also been implicated (8, 16, 26). Certainly, phenotypically similar campylobacters have been isolated (26) from both drinking water and birds in the same broiler unit but it is unclear whether the water infects the birds or vice versa, although several studies (8, 18, 19) have shown that water contamination usually follows flock infection.

Intervention measures to prevent *Campylobacter* colonization of birds during broiler production are crucial for the control and prevention of human disease. *Campylobacter* presence in the drinking water of broiler houses may be indicative of a failure in overall biosecurity and may be due to infected source water, ineffective cleaning procedures, effective cleaning procedures incorrectly applied, or poorly designed delivery systems. The aims of this study were to investigate the *Campylobacter* status of broiler drinking water, in particular, the water in header tanks feeding the drinker delivery system. Sequence-based typing methods were used to compare the genotypes of isolates found in water to those from broiler chickens in the same shed.

MATERIALS AND METHODS

Farms. Twelve broiler farms located within a 25-mile (40-km) radius of each other in northeastern Scotland were studied. All of the farms were visited at least twice. The first visit to each farm occurred within a 6-month period in the winter and spring of 1999 and 2000. A second round of sampling was delayed due to the foot-and-mouth disease (FMD) outbreak in 2001, during which farm access was suspended. Two of the farms were tested twice during the first testing period. Most of the second round of sampling took place approximately 12 months later (post-FMD). On this second visit, additional sampling was carried out on eight of the farms.

The water source (e.g., water main or private supply) for each farm was

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▽ Published ahead of print on 22 June 2007.

recorded. Those with private supplies treated their header tank water intermittently (records unavailable) with quaternary ammonium biocides during the rearing of flocks. Between flocks, the same biocide was allowed to stand overnight in the delivery pipes prior to flushing with clean water. Individual nipple drinkers were not dismantled for cleaning. Farms comprised several sheds, some being adjacent to each other and fed from a common water storage (header) tank. Other sheds were spatially separate and fed by independent tanks. Not all sheds were populated with chickens, and where possible, the shed sampled contained birds >30 days of age.

Sampling. Samples to detect colonization in birds were taken from cloacal swabs or fresh feces from litter or cull birds. Water samples were taken from nipple drinker surfaces with swabs, from the end of drinker distribution (feed) lines, and from header tanks. Feed lines were sampled (after sterilization of surfaces with 70% ethyl alcohol) by draining the end of the horizontal delivery pipe. Most of the header tanks were sited within the eaves of the poultry shed (some were fed from adjacent shed tanks), had a holding capacity of approximately 500 to 1,000 liters, and were constructed of plastic with loosely fitting lids. Collection of water samples (5 liters) from these tanks was done by means of a manual siphon and was performed aseptically after immersion of the siphon in 70% ethyl alcohol, followed by rinsing with sterile distilled water. All samples were taken in quintuplicate and immediately transported in cool boxes to the laboratory for analysis on the day of collection.

Isolation of campylobacters. To optimize *Campylobacter* recovery from water samples, a modified version of the method of Slader et al. (30) was used where samples (5 liters) were filtered (0.22-μm pore size) and the filter disks were enriched microaerobically (100-ml volumes of nutrient broth base [Mast, Bootle, United Kingdom] with 5% horse blood, growth supplement [Mast Selectavial SV61], amphotericin [2 μg/ml], cefoperazone [15 μg/ml], and trimethoprim [10 μg/ml]) at 37°C. After 6 to 8 h of enrichment, two additional antimicrobials (polymyxin B [2,500 IU/liter] and rifampin [5 μg/ml]) were added to the broths, which were then cultured for a further 5 days. All antimicrobials were purchased from Sigma-Aldrich United Kingdom. Enrichment broths (0.1 ml) were plated, after 2 and 5 days, on charcoal cefoperazone deoxycholate (CCD, CM0739; Oxoid, United Kingdom) agar incubated microaerobically at 37°C. Swabs (cloacal and drinker surfaces) and feces (25 g) were enriched as described above. Colonies were presumptively identified as *Campylobacter* spp. microscopically (Gram staining) and by agglutination with Microscreen latex (Microgen, Camberley, United Kingdom). Individual colonies (five from each sample) were stored (−80°C, nutrient broth plus 15% glycerol) for genotypic analysis.

Genotyping. A total of 227 strains were genotyped, including multiple colonies from single samples. Isolates were plated frozen onto CCD agar and incubated microaerobically for 48 h at 37°C. Bacterial DNA was prepared by making a suspension of freshly grown bacterial cells in 125 μl of phosphate-buffered saline (Sigma Aldrich Company Ltd., Dorset, United Kingdom), vortexing them briefly, and immediately incubating them at 100°C for 10 min. The suspension was clarified by centrifugation at 13,000 × *g* for 10 min, and the supernatant was stored at −20°C.

Campylobacter isolates were identified to the species level by a modification of the method and primers described by Wang et al. (33). Each multiplex PCR mixture contained 0.2 μl of a 10 mM deoxynucleoside triphosphate mixture (Invitrogen Ltd., Paisley, United Kingdom), 1 μl of 10× reaction buffer (QIAGEN Ltd., Crawley, United Kingdom), 0.05 μl of *Taq* polymerase (QIAGEN), 0.2 μl of a 10 μM primer mixture containing *Campylobacter jejuni* and *Campylobacter coli* primers, 0.4 μl of chromosomal DNA, and molecular biology grade water (Sigma Aldrich Company Ltd.) to a final volume of 10 μl. The reaction conditions were an initial incubation at 95°C for 2 min, followed by 30 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s, with a final extension step of 72°C for 5 min. Amplicon sizes were compared against molecular weight markers (Hyperladder IV; Bioline, London, United Kingdom) on a 2% agarose gel.

Multilocus sequence typing (MLST) was carried out by the method of Dingle et al. (6) with the use of additional primers described by Miller et al. (21). Each 25-μl PCR mixture contained 0.5 μl of 10 mM deoxynucleoside triphosphates (Invitrogen Ltd.), 2.5 μl of 10× reaction buffer (QIAGEN Ltd.), 0.125 μl of *Taq* polymerase (QIAGEN Ltd.), 0.5 μl of each 10 μM primer, and 2 μl of chromosomal DNA. Amplification conditions were 95°C for 2 min, followed by 35 cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 1 min, followed by an extension step of 72°C for 5 min. Amplification products were purified by 20% polyethylene glycol–2.5 M NaCl precipitation (7) and sequenced in each direction with primers identical to those used for PCR. Sequencing reaction mixtures (10 μl) contained 0.25 μl of BigDye ReadyReaction Mix (Version 3; Applied Biosystems, Foster City, CA), 1.87 μl of 5× sequencing buffer (Applied Biosystems), 4 μl of 0.67 μM primer, 1.5 μl of template DNA, and 2.38 μl of molecular biology grade water (Sigma Aldrich Company Ltd.). Reaction conditions of 96°C for 10 s, 50°C for 5 s, and 60°C for 2 min for 30 cycles were used, and sequencing products were resolved with an ABI 3730 automated sequencer (PE Biosystems). Sequences were assembled with STARS software available at http://pubmlst.org, and newly identified alleles and sequence types (STs) were submitted to the *Campylobacter* MLST database at this website.

TABLE 1. Summary of results of the first farm sampling showing water sources and STs of isolates recovered from chickens and drinkers

Farm	Testing date (mo, yr)	Source of broiler shed water	Genotype(s) (STs) of isolate(s) from:	
			Chickens	Drinkers
A	November 1999	River	573, 137	573
A	May 2000	River	267, 573	267, 61, 45
B	May 2000	Borehole	50	50
C	March 2000	Water main	50, 573	50, 573
D	April 2000	Water main	50	50
E	April 2000	Water main	573	—[a]
F	March 2000	Hill runoff	573	267
H	April 2000	Water main	50	—
I	April 2000	Water main	50	50
J	November 1999	River	50	50
J	May 2000	River	267	61, 267
K	May 2000	Water main	50	—
L	March 2000	Water main	53, 573	573
M	April 2000	Water main	50	573

[a] —, no campylobacters isolated.

RESULTS

Of the 12 farms tested, 8 were supplied by treated water main water while the rest were supplied with untreated water from rivers, a borehole, or hill runoff. Presumptive campylobacters were most often confirmed from bird samples after the 2-day enrichment period, whereas target isolation from water samples required the extended 5-day enrichment. We observed great diversity in confirmed *Campylobacter* morphology on CCD agar (data not presented), although many *Campylobacter*-like colonies were not confirmed as such by PCR. Campylobacters were isolated from the birds at all 12 of the farms tested before the FMD outbreak (Table 1). All but three of the shed drinker systems were contaminated with recoverable campylobacters. The *Campylobacter*-negative water sources were all water main fed. MLST confirmed 97% of the isolates recovered as *C. jejuni* and the remainder as *C. coli*. Multiple STs were recovered from individual bird samples and single 5-liter water samples on several occasions. The STs of the isolates recovered from the drinkers matched those of the isolates from birds in the same sheds on 9 of the 14 testing occasions. Two STs were recorded at multiple farms, i.e., ST 50 at eight farms and ST 573 at six.

During the second round of testing, more comprehensive sampling was undertaken, in particular to investigate *Campylobacter* contamination of the header tanks in each poultry shed (Table 2). Four of the eight farms tested (farms A, E, F, and J) had recoverable campylobacters in the header tanks. At farm J, this contamination was widespread, with contamination in the header tanks in three of the four operational sheds. Farms B, C, K, and M all had campylobacters in the birds, but none were recovered from the header tanks. In farms A, E, and F, the STs of the strains from header tanks differed from those

TABLE 2. Genotypes of *Campylobacter* isolates from poultry and broiler shed header tanks determined at second farm sampling

Farm	Testing date (mo, yr)	Shed no.	Genotype(s)[a] (STs) of isolate(s) from:	
			Chickens	Header tank
A	May 2000	3	573	267
B	February 2002	4	NT	—
C	February 2002	1	NT	—
E	December 2001	1, 2, 3, 4	5	573
F	February 2002	3	45	230, 1674
J	March 2002	5	51, **267**	137, **267,** 1673
J	March 2002	6	**53, 267**	51, **53, 267**
J	March 2002	7	51, 53, **573**	**573**
K	February 2002	1, 2	NT	—
M	November 2001	8	NT	—

[a] NT, *Campylobacter* positive but not tested by MLST. —, *Campylobacter* negative. Bold type indicates matching STs between birds and the header tank in the same broiler shed. The header tank at farm E fed all four sheds.

of the strains carried by birds in the same shed. For example, farm E showed a consistent ST (ST 5) in strains from birds in all four sheds but only an isolate of ST 573 was found in the common tank supplying these sheds. At farm F, shed 3 had two different isolates in the header tank (STs 230 and 1674) but a strain of ST 45 colonized the birds. However, farm J had isolates of matching STs in both the birds and header tanks in all three of the sheds tested. Interestingly, in shed 5 of farm J, there were two STs detected in the birds (STs 51 and 267) but only one matched the three different STs in the header tank (STs 137, 267, and 1673). Shed 6 also had strains of two different STs in the birds (STs 53 and 267) and three different STs in the water (STs 51, 53, and 267), while shed 7 had isolates of three different STs in the birds (STs 51, 53, and 573) and a matching ST in the water (ST 573).

DISCUSSION

All 12 farms contained *Campylobacter*-positive birds on all of the occasions tested, indicating a very high prevalence of flock colonization. In this study, colonization appeared to be independent of whether the water came from a treated water main or from untreated supplies. From our investigations, it is clear that when birds are colonized, drinker surfaces frequently become contaminated, potentially enabling extensive cross-contamination and rapid transmission throughout the house. This is not surprising, given the evidence of palatine colonization with campylobacters in birds (22). However, the involvement of header tanks in the epidemiology of *Campylobacter* infection in broilers has not previously been investigated. The results of our studies, using a molecular epidemiological approach, clearly indicate that this is feasible at at least one (farm J) of the 12 farms studied. Unfortunately, given the structure of this study, is was not possible to establish the sequence of contamination and infection events at farm J; i.e., was the header tank positive before the birds, or did the infected birds contaminate the header tank? However, either scenario would be potentially important in the control and prevention of poultry flock colonization.

The most obvious source of *Campylobacter* contamination of the drinkers is colonized birds, with the bacterium tracking back along the feed lines to the header tank. However, despite the highly efficient mobility of campylobacters mediated by the characteristic bipolar flagella, tracking back (over a distance of at least 12 feet [4 m]) against gravity and water flow and in the absence of a chemotactic stimulus seems unlikely. This is supported by the observation that only 1 of the 12 farms (farm J) with colonized flocks demonstrated this association. The header tank was fitted with a lid, but it is also a possibility that its contents were contaminated by campylobacters from within the shed from nonwater vectors, e.g., airborne spread (2) or carriage by insects (13). However, farm J was tested on 11 March 2002, when the ambient temperatures were 0.7 to 8.8°C (http://www.metoffice.gov.uk/), which would have been unsuitable for insects and, although internal shed temperatures were higher, there was no evidence of insect life. Human intervention could also result in contamination of the tank with campylobacters from the flock within the matching poultry house. In the case of farm J, discussions with farm staff revealed a routine manual addition of biocides to the drinking water via the header tanks during flock placement, so this cannot be discounted as a route of contamination. Although such a contaminated header tank will have no implications for the already colonized flock in the house, in the case of shared header tanks (e.g., farm E, Table 2), it could result in the transmission of strains between adjacent houses (although this is more likely to occur directly via, e.g., farm staff). Moreover, if survival was long term, then this could provide a residual source for subsequent flocks in the same house. Interestingly, previous investigations (29) indicate that the carryover of strains from one flock to a subsequent flock in the same house is a relatively rare event, occurring in less than 10% of 100 houses studied, which is very similar to the incidence of matching flock and tank contamination seen here (1 of 12 farms).

An alternative source of *Campylobacter* contamination of the drinkers is that the water entering the header tank is contaminated and, in turn, leads to colonization of the flock. The extraction point for the water supply to farm J is a local river that has a mean flow of 8.77 $m^3 s^{-1}$ (http://www.sepa.org.uk/data/river_levels/data.htm) and receives untreated drainage from multiple ruminant and pig farms, some immediately upstream of farm J. Furthermore, the farm is approximately 2 miles (3 km) downstream from a sewage works serving a town of approximately 5,000 people. It is likely, therefore, that campylobacters from several sources intermittently contaminate the river. The survival of *C. jejuni* in fresh water is temperature dependent (25). The approximate temperature of rivers in northeastern Scotland during March is 4°C, and at this temperature campylobacters should survive for up to 7 days (25), which would provide ample opportunity for viable riverborne organisms to contaminate the header tanks. The observation of multiple strain types in the header tank of farm J indicates that such contamination is repeated and frequent.

Clearly, once campylobacters enter header tanks, they can survive in this environment for short time periods. However, such a water environment would be hazardous long term as they would be exposed to nutrient deprivation, high oxygen, and low osmolarity. These organisms have few reported mechanisms for stress regulation, but their recently identified capacity to survive in both biofilms (27, 32) and protozoa (31) may enable persistence in header tanks. Nevertheless, the mo-

lecular epidemiological observations in our study raise a number of issues regarding water as a significant risk factor for *Campylobacter* colonization in poultry flocks. In particular, it is notable that although 50% of the farms tested had recoverable campylobacters in their header tanks, in only one of these was the same strain found in the flock. There are several possible explanations for this. Firstly, the recovery of campylobacters from water is difficult even with current sensitive enrichment techniques (the extended 5-day enrichment was required for positive isolation from header tank water) and the fragility of these organisms under such environmental stresses is probably strain dependent, so it is possible that not all of the contaminating strains were recovered. In addition, farm testing was carried out usually only once in a flock cycle and although 5-liter volumes were tested, filtration was frequently difficult because of particulate matter; therefore, the results probably underestimate the extent of the problem. Secondly, under such conditions, the dose of campylobacters capable of chicken colonization may be insufficient. A dose of as few as 10 organisms freshly derived from humans or chickens may colonize young birds (3, 4). The daily consumption of water by birds >30 days of age is approximately 250 ml (35), which is equivalent to 6,250 liters daily in sheds holding 25,000 birds (e.g., farm J). Given that older birds drink 250 ml of water daily, header tanks with a 1,000-liter capacity serving large flocks of older birds would be replenished continuously. Source water is likely to be at a lower temperature than the broiler shed, and contaminating campylobacters are therefore likely to be viable (25), thus increasing the chances of infecting individual birds. Broiler contamination via this route only requires the infection of one bird, as a single in vivo passage will result in up-regulation of colonization potential (3) and subsequent fecal-oral transmission will cause rapid and widespread flock infection (29). However, environmental stress, such as exposure to water (10) and aerobic conditions (12), can cause a reduction in colonization potential and this effect may also be strain dependent (5).

The *Campylobacter* genome is highly plastic, and many multiple typing methods have been developed and used for this organism. Although MLST was initially designed for the study of population evolutionary trends (20), it is now widely used in the United Kingdom as a genotyping tool. The number of isolates typed within our study is insufficient to draw conclusions regarding epidemiological relationships between farms or comparisons with other studies, although common STs were observed at multiple farms within short time frames (Table 1). However, with such a tool the identification of clonal relationships to physiological characteristics of the pathogen may be feasible. A previous study (11) has indicated that some clonal complexes (e.g., the ST 45 complex) are preferentially recovered from water sources like rivers. This suggests that survival in water may be a genetically determined characteristic in campylobacters. In our study, isolates representing a wide variety of clonal complexes were recovered from the header tanks, including complexes 573, 283, 45, 443, and 21. Interestingly, clonal complex 45 has been previously highly associated with water environments in another epidemiological study (11) and this may support suggestions that some campylobacters have evolved stress response systems enabling effective persistence in water (17).

The results of this study present genotypic evidence that drinking water may be associated with *Campylobacter* infection in some broiler flocks. The importance of this source compared with other potential sources is difficult to assess, but it may be the causative route in approximately 10% (here it was 1 in 12 farms, 8.3%) of flocks and is therefore a practical target for intervention. This is consistent with a previous study (16) that indicated that water sanitization can significantly reduce the prevalence of flock positivity at slaughter. Possible practical intervention strategies which have been tested with positive results (16, 26) include the replacement of untreated private water supplies with public water main water (although in rural areas this may be costly) and the installation of chlorine dose meters and UV treatment systems.

ACKNOWLEDGMENTS

This study was funded in part by the Food Standards Agency, United Kingdom, under project BO3010. All typing data were funded by DEFRA project OZO611, Great Britain. D. G. Newell was also supported by DEFRA under project FZ2100.

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