

Campylobacter in Waterfowl and Aquatic Environments: Incidence and Methods of Detection[†]

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*Campylobacter*s are emerging as one of the most significant causes of human infections worldwide, and the role that waterfowl and the aquatic environment have in the spread of disease is beginning to be elucidated. On a world scale *Campylobacter*s are possibly the major cause of gastrointestinal infections. *Campylobacter*s are common commensals in the intestinal tract of many species of wild birds, including waterfowl. They are also widely distributed in aquatic environments where their origins may include waterfowl as well as sewage effluents and agricultural runoff. *Campylobacter*s have marked seasonal trends. In temperate aquatic environments they peak during winter, whereas spring–summer is the peak period for human infection. *Campylobacter* species may survive, and remain potentially pathogenic, for long periods in aquatic environments. The utility of bacterial fecal indicators in predicting the presence of *Campylobacter*s in natural waters is questionable. Viable but nonculturable *Campylobacter* cells may occur, but whether they have any role in the generation of outbreaks of campylobacteriosis is unclear. The routine detection of *Campylobacter* spp. in avian feces and environmental waters largely relies on conventional culture methods, while the recognition of a particular species or strain is based on serotyping and increasingly on molecular methods. Thus, PCR combined with selective enrichment enhances the detection of *Campylobacter*s in water and feces, while DNA sequencing facilitates recognition of particular species and strains.

Introduction

Gastroenteritis remains the leading cause of morbidity and infant death in developing countries (1). A wide range of microorganisms is responsible for human gastroenteritis, these include viral, protozoan, and bacterial pathogens. Among bacteria, *Salmonella* has been considered, for many years, as the commonest cause of bacterial diarrhoea infection in humans worldwide (2). However, in the last two decades, *Campylobacter* spp. have been recognized as the possible leading cause of bacterial gastroenteritis in humans in both

industrialized and developing countries (3–5). The majority of *Campylobacter*-associated infections are thought to be sporadic cases of food poisoning, while contaminated drinking water, including that from public water supplies, is believed to be the vehicle of large *Campylobacter*-associated outbreaks (6, 7). Although *Campylobacter* spp. are widely distributed in the environment, the epidemiology of many cases of *Campylobacter*-associated infections remains unclear. This is probably because of the lack of routine, parallel typing of isolates from the environment and those from clinical cases (5, 8). This paper aims to review the incidence of *Campylobacter* spp. in waterfowl, which are a highly mobile and potentially important source of *Campylobacter*s, and in aquatic environments. Methods of detection and confirmation are also discussed.

Campylobacter in Waterfowl

Waterfowl and wild birds in general are regarded as attractive and as indicators of a healthy environment (9). However, they also might be viewed from a public health stand point as polluters and carriers of infectious agents (8, 10).

Waterfowl have been reported as carriers of a wide range of pathogenic viral, bacterial, fungal, and parasitic microorganisms (11, 12). Among bacterial pathogens, *Campylobacter* species have been frequently isolated from the feces and/or cloacal swabs of apparently healthy ducks and geese worldwide (12–26). *Campylobacter jejuni*, which is responsible for most *Campylobacter* infection in humans, was found to be more prevalent in the feces of waterfowl than *C. coli* and *C. lari* (12, 15–17, 24, 26, 27).

The carriage of *Campylobacter* species in waterfowl has frequently been reported in healthy birds without obvious symptoms of diarrhea, including the absence of gut lesions associated with *Campylobacter* infection; this was also observed in other birds (i.e. gulls, pigeons, crows) (13, 28, 29, 30). These observations suggest that there is a nonharmful coexistence between *Campylobacter*s and their bird hosts (13, 29, 30). Thus, *Campylobacter*s inhabiting the intestinal tract of waterfowl and other wild birds are regarded as commensals (13, 30). The body temperature of waterfowl, and wild birds in general, is around 42 °C which is the optimal growth temperature of thermotolerant *Campylobacter*s (i.e. *C. jejuni*, *C. coli*, *C. lari*, and some strains of *C. upsaliensis*). This might explain the high prevalence of *Campylobacter* species in waterfowl albeit without clinical manifestations (13, 29, 30).

Feeding habits of waterfowl may, in part, play a role in the carriage rate and overall incidence of *Campylobacter*s in their intestinal tract. Green-winged teal duck (*Anas acuta*)

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TABLE 1. Examples of Campylobacters in Environmental Waters^a

sample type	location	N:P (%)	species identified	recovery method	refs
river, ground, and drinking water	New Zealand	42:27 (64)	not determined	MPN and PCR	37
drinking water and groundwater	Finland	20:8 (40)	<i>C. jejuni</i> , <i>C. coli</i>	MFE	38
river water	Lancashire, U.K.	312:134 (43)	<i>C. jejuni</i> , <i>C. coli</i> , <i>C. lari</i>	MFE and MPN	39
pond water	Hull and East Yorkshire, U.K.	211:98 (46)	<i>C. jejuni</i> , <i>C. coli</i> , <i>C. lari</i>	MFP	52
pond water	East Yorkshire, U.K.	54:19 (35)	<i>C. jejuni</i> , <i>C. coli</i>	MFE	53
seawater	Valencia, Spain	192:25 (13)	<i>C. jejuni</i> , <i>C. coli</i>	MFE	55
seawater	Lancashire, U.K.	GM ^b	<i>C. lari</i> , UPTC	MPN	56
runoff water	East Yorkshire, U.K.	36:7 (19)	<i>C. coli</i>	MFE	26
lake and pond water	Central Washington, U.S.A.	99:41 (41)	<i>C. jejuni</i> , <i>C. coli</i>	MFE, MPN	50
lake water	Greece	86:14 (16)	<i>C. jejuni</i>	MFP	42
groundwater	Lancashire, U.K.	72:12 (17)	<i>C. jejuni</i>	MFE	54
river water	Norway	60:32 (53)	<i>C. jejuni</i> , <i>C. coli</i> , <i>C. lari</i>	MFE, MFP	45
river water	Norway	not determined	<i>C. jejuni</i> , <i>C. coli</i>	PCR ^c	117
stream and drinking water	New Zealand	293:162 (55)	<i>C. jejuni</i> , <i>C. coli</i>	PCR ^c	24

^a Abbreviations: MPN = most probable number, MFE = membrane filtration and selective enrichment followed by selective plating, MFP = membrane filtration and selective plating of the filters, UPTC = urease-positive thermophilic campylobacters, N = total number of samples; P = total number of positive samples; (%) = percentage of positive samples. ^b GM = geometric means given (GMs for 12 batches of 30–60 samples ranged from 3 to 148 per 100 mL). ^c PCR detects presence or absence after selective enrichment.

that usually feed on vegetation had a carriage rate of 16%, compared to 66% in shoveler duck (*Spatula clypeata*) that usually strain bottom sediments of aquatic environments to extract molluscs (13). Similarly gulls and crows had higher carriage rates of *Campylobacter* spp. than pigeons (28, 30, 31). Gulls and crows often scavenge on refuse tips and sewage sludge, whereas pigeons are herbivorous (30, 31).

In contrast to the several reports describing a high prevalence of *Campylobacter* spp. in the intestinal tract of waterfowl, Hill and Grimes (32) did not isolate the organism from around 50 avian caecal contents as well as from the water and sediment of Lake Onalaska, WI. This lake (area 3144 ha) harbored around 619 000 ducks and geese during autumn. Similarly, Feare et al. (33) failed to detect thermotolerant campylobacters from around 600 fecal droppings of ducks and geese collected from 12 sites in London, southeast England, Yorkshire, and northern England over a period of 2 years. These findings suggest that thermotolerant campylobacters may exhibit a sporadic distribution among waterfowl (32, 33), perhaps as a result of feeding habits and seasonal variation. Alternatively, the apparent absence may be due to problems with the methods used to detect these bacterial species in fecal samples and/or cloacal swabs.

Campylobacter in Aquatic Environments

Occurrence. Campylobacters are exogenous to aquatic environments. So far, there is no available evidence that *Campylobacter* species are capable of growing in the environment, i.e., other than in warm-blooded hosts or laboratory media. This is probably because of their high minimal growth temperature (>30 °C) (5, 34, 35). Thus, the presence of campylobacters suggests recent fecal contamination (9, 35). Aquatic environments that receive fecal contamination should be regarded as potentially contaminated by *Campylobacter* spp (36). Indeed, *Campylobacter* species have been isolated from fecally contaminated drinking water (37, 38), rivers (37, 39–49), lakes (42, 50, 51), ponds (50, 52, 53), drainage channels (52), groundwater (37, 38, 54), marine waters (48, 55, 56), and runoff water (26) worldwide (Table 1).

The diversity of *Campylobacter* populations in aquatic environments is dependent on sources of contamination. The River Calder, Lancashire, U.K., received discharge from a sewage works and *Campylobacter jejuni* was the predominant species, whereas *C. coli* and *C. lari* were more prevalent in the geographically close River Ribble as a result of fecal contamination from birds (gulls) or runoff from nearby pig farms (39). Pollution in the River Lune, northwest England

was found to be a mixture of sewage effluents, agricultural runoff, and fecal deposits from large flocks of waterfowl. Here *C. jejuni*, *C. coli*, *C. lari*, and urease-positive thermotolerant campylobacters (UPTC) were all isolated (44). At a marine site, Morecambe Bay, England, although a sewage treatment plant discharged effluent into the bay, campylobacters recovered from the water comprised *C. lari* and UPTC only. Their source was suggested to be the large flocks of gulls, knots, and oystercatchers harbored within Morecambe Bay (56). In village ponds in northeast England duck populations and runoff water influenced the *Campylobacter* species found. In one pond, genetically similar strains of *C. jejuni* were recovered from pond water and sediment and also from duck feces. Runoff from an adjacent road tested negative for campylobacters, thus ducks seemed to be the source of the organism (26). In contrast, although ducks were present in the vicinity of other ponds, genetically indistinguishable strains of *C. coli* were isolated from runoff water and from pond water and sediment but not from duck feces, suggesting that the ducks were not the source of *C. coli* (26).

Survival and Seasonality. Campylobacters in water survive for longer at lower temperature (57–60), with decay constants *k* of –0.05 d⁻¹ or less at 4–5 °C (57, 59), perhaps explained by increased metabolism and more rapid substrate utilization at higher temperature at the expense of culturability (59). Thus a culturable population of *C. jejuni* in sterile streamwater (10⁴ cfu mL⁻¹) survived for more than 4 months at 4 °C (*k* = –0.04 d⁻¹) (59). In addition, light (particularly UV radiation) is potentially lethal to campylobacters in aquatic environments (58). In laboratory experiments, natural populations of culturable thermotolerant campylobacters, in sea and river water, decreased within less than 10 min exposure to simulated summer sunlight, and none could be recovered after 30 min (58). Light affects the uptake of nutrients and inhibits active transport and biosynthesis in *E. coli* (61); its effects on *Campylobacter* spp. require further investigation (62).

Perhaps in response to seasonal change in temperature and solar radiation, campylobacters in temperate environmental waters tend to show strong seasonal variation in their abundance. The organism is frequently abundant during late autumn and winter but is less so or absent in summer. This general pattern has been reported from studies of rivers (39, 41, 44), lakes (41, 50, 51), ponds (52), and coastal seas (55, 56).

Conflicting reports on *Campylobacter* seasonality have, however, come from Greece, where no statistically significant differences in the occurrence of campylobacters, in lake and

TABLE 2. Correlations between Abundance of Campylobacters and Abundance of Fecal Indicators in Aquatic Environments

fecal indicators	location of samples	type of sample	correlation	refs
total coliforms	Germany	river water	yes	40
fecal coliforms, fecal streptococci, sulfite reducing clostridia	Norway	lake and river water	yes	41
total coliforms, fecal coliforms	Poland	lake and river water	yes	46
fecal coliforms, fecal streptococci	U.K.	river water	no	44
total coliforms, fecal coliforms, fecal streptococci	U.S.A.	lake, river, and pond water	no	50
total coliforms, fecal coliforms, fecal streptococci	Greece	lake and river water	no	52
thermotolerant coliforms, <i>E. coli</i> , <i>Clostridium perfringens</i>	Finland	lake and river water	no	47
total coliforms, fecal coliforms	Spain	marine water	no	55
fecal coliforms, fecal streptococci	U.K.	marine water	no	56

river waters, were observed between warm and cold months (42). Likewise, in the River Odra and Szczecin Lagoon in Poland, the presence or absence of *Campylobacter* species was not significantly related to water temperature (46). Also, Eyles et al. (49) found that campylobacters in the Taieri River, New Zealand were more abundant during summer than winter and suggested that continuous fecal contamination coupled with minimum dilution during low summer river flow might explain the observed trend.

Seasonal patterns in *Campylobacter* abundance have also been observed in sewage effluents. Unlike in natural waters, campylobacters in sewage effluent may peak in summer (63, 64) in response to infection rates in the human community and to *Campylobacter* levels in slaughter house waste (63). However, a different seasonal picture for *Campylobacter* spp. in sewage effluent was observed by Koenraad et al. (65) at three municipal wastewater plants in The Netherlands where peak levels were observed at diverse times of year and where the generally lesser abundance during June–August may be related to reduced survival at higher environmental temperature (65).

Predation and nutrient availability are also important factors in the survival of enteric bacteria in natural waters (66). Survival of campylobacters was better in lake water that had been filtered through 0.2 µm cellulose nitrate filters than in unfiltered lake water due to the removal of zooplankton grazers and reduced competition for nutrients; for example, the survival of culturable *C. jejuni* was about 2 orders of magnitude greater in filtered water after 10 days at 4 °C (67). Also, culturable *C. jejuni* survived for over 20 days at 4 °C in nonautoclaved, 0.2 µm-filtered, lake water compared to <10 days in similar but autoclaved water, suggesting that autoclaving had destroyed heat-labile nutrients required by campylobacters or possibly produced toxic byproducts that may have affected the cells (68). This finding was not observed with *Escherichia coli* which, in autoclaved lake water at both 4 °C and 25 °C, showed little loss of culturability over about 40 days, whereas *C. jejuni* became unculturable within <10 days (68).

There may be differences in the survivability of different *Campylobacter* species in environmental waters; this is important because *C. jejuni*, among the diverse species found in the environment, is the major cause of human *Campylobacter* infections. Comparative laboratory studies have given conflicting results. Korhonen and Martikainen (67) found that *C. jejuni* survived longer than *C. coli* in both unfiltered and 0.2 µm-filtered lake water at both 4 °C and 20 °C; for example, after 10 days at 4 °C in unfiltered water the abundance of culturable *C. jejuni* was about 2 orders of magnitude greater than that of *C. coli*. Similarly, Thomas et al. (57) observed that *C. jejuni* survived better than *C. coli* and *C. lari* under a diversity of conditions; i.e. in sterile deionized water; sterile river water; sterile river water with sediments; held at 5 °C, 15 °C, 25 °C, and 37 °C (sterilization of water and sediment suspensions were by autoclaving). Thus, for example, *k* values for *C. jejuni* at 5 °C were –0.02 to –0.04 d⁻¹ compared to –0.06 to –0.07 d⁻¹ for *C. coli* and

–0.05 to –0.06 d⁻¹ for *C. lari*. In contrast, Obiri-Danso et al. (58) found that cultures of *C. jejuni* and *C. coli* had similar survival times in artificial seawater and that both these species survived less well than *C. lari* and UPTC. Thus survival times to nonculturability, in darkness at 4 °C, were 312–336 h for *C. jejuni* and *C. coli* compared to 384–600 h for *C. lari* and UPTC. Conflicting results from different studies may, in part, be explained by variability between strains, differences in experimental systems, and growth history of the organisms (69).

Correlation with Fecal Indicators. The use of fecal indicators (fecal coliforms, *E. coli*, fecal streptococci, *Clostridium perfringens*) aims to evaluate water sources intended for supply or recreation, by predicting the presence of pathogenic microorganisms. Significant correlations have been found between total coliforms and campylobacters in river waters that receive fecal contamination from waterfowl and poultry farms in Germany (40). Similarly, Brennhovd et al. (41) found highly significant separate regressions between the abundance of three indicators (fecal coliforms, fecal streptococci, and sulfite-reducing clostridia) and the abundance of campylobacters in fresh waters (Lake Østensjøvannet, Lake Sognsvann, and the River Nitelva, Norway). In Poland, the presence or absence of *Campylobacter* species in surface fresh waters was related to total coliforms and fecal coliforms (46). Together, these studies suggest that fecal indicators are potentially a useful warning of the potential presence of *Campylobacter* spp. in aquatic environments (Table 2).

However, relationships are not always found between fecal indicators and campylobacters (Table 2); an observation that may be related to campylobacters being in a viable but nonculturable state (see the later VBNC section). Obiri-Danso and Jones (44) found poor correlations between campylobacters and fecal coliforms in river water in northwest England during a 2-year study although fecal streptococci were correlated with campylobacters but only in 1 year. Carter et al. (50) isolated campylobacters from lakes, ponds, and mountain streams in central Washington, U.S.A., but their density was not significantly correlated with fecal indicators. Also, in lake and river waters in Greece, counts of total coliforms, fecal coliforms, and fecal streptococci did not differ significantly between *Campylobacter* positive and negative samples (42). The presence or abundance of *Campylobacter* often seems not to be related to abundance of fecal indicators. This was observed in fresh waters in Finland (47) and marine waters in Spain (55) and England (56). Thus, the ability of fecal indicators to predict the presence of campylobacters in environmental waters remains questionable, and the absence of fecal indicators is not a reliable indication of the absence of *Campylobacter* spp.

The Viable but Nonculturable State. Viable but nonculturable (VBNC) in the context of enteric bacteria refers to the ability of bacterial cells to remain viable (i.e. retaining basal metabolic activity) yet unable to grow in artificial media (70). This state is believed to be a survival strategy of enteric bacteria that are released into the environment and suffer

prolonged exposure to environmental stressors (e.g. sub-optimal temperature; UV irradiation; nutrient deprivation; biological interactions) (71–73). The VBNC state was described by Xu et al. (74) who examined the survival of *Vibrio cholerae* and *E. coli* in estuarine and marine water microcosms. The VBNC state is exhibited by *Campylobacter* spp. and by many other bacteria; e.g. *Salmonella*, *Shigella*, *Legionella* (59, 71). Survival strategies of *C. jejuni*, including adoption of the VBNC state, in response to diverse agents of stress in the environment are reviewed by Murphy et al. (75).

Nonculturability in campylobacters has been attributed, in part, to exposure to high temperature (59, 76); other relevant factors may include nutrient depletion, salinity, and aeration (77). VBNC forms of *Campylobacter* spp. undergo morphological and physiological transitions in response to stress. Morphological changes include the formation of elongated spirals, rods, and coccoid forms, while physiological changes include reduction in intracellular ATP concentration and loss of cytoplasmic membrane fatty acids (59, 62, 76). Coccoid forms have been associated with the VBNC state; Hudock et al. (78), however, concluded that spiral-shaped cells in *C. jejuni* are candidates for the VBNC state, whereas the formation of coccoid cells was associated with degradation of DNA and of cells. Resuscitation of VBNC *Campylobacter* spp. may be possible. Viable but nonculturable forms of *C. jejuni* were able to colonize suckling mice, the gut of rats, 1-week-old chicks, and fertilized chicken eggs (79–82). Indeed, Baffone et al. (83) were able to resuscitate *C. jejuni*, after up to 142 days as VBNC cells in artificial seawater, by passage through mouse intestine. This suggests that *C. jejuni* in VBNC form may potentially retain virulence and the capability of causing infection. Whether VBNC cells from the environment are able to cause infection without prior resuscitation remains unclear (72).

In contrast, other studies have rejected the whole VBNC concept. It is suggested that VBNC cells are actually dead and that apparent resuscitation represents the growth of surviving culturable cells (84–86). This suggestion was supported by failure of VBNC *Campylobacter* to colonize 1-day-old chicks and by nonresuscitation in simulated colon environments (87–89).

Although the VBNC phenomenon is not fully elucidated, it has a major relevance to public health authorities in two areas: i.e. the ability to cause infection and the monitoring of enteric pathogens in environmental samples by conventional culture methods (71, 90).

Detection of *Campylobacter*

Detection in Waterfowl. Direct inoculation of avian fecal samples and/or caecal contents onto selective agar plates has frequently been used for the detection of campylobacters (13, 15, 19, 21–23, 28, 30, 31). Other studies employed a combination of selective enrichment in broth culture followed by selective plating (14, 26, 29). Although direct inoculation of human feces onto selective agar is good for the recovery of campylobacters from diarrhea patients (where high numbers of the target organism are present) (91), it might not guarantee a high recovery rate from feces of animal or avian origin. Thus, selective enrichment followed by selective plating potentially increases recovery rates of campylobacters (92, 93). The use of direct plating without prior enrichment perhaps contributed to the failure of Hill and Grimes (32) to detect campylobacters in waterfowl caecal contents and to the low incidence of *Campylobacter* spp. (2%) found by Palmgren et al. (94) in feces and rectal swabs of gulls and diverse species of small birds.

The choice of media and incubation temperature also plays an important part in the detection of campylobacters in feces. Comparative studies that tested the suitability of different enrichment and plating media for the recovery of

campylobacters from feces suggested that Preston enrichment broth, Preston agar and modified charcoal, cefoperazone, and deoxycholate agar (mCCDA) were to be preferred because they gave fewer problems with contaminant microflora (91, 92, 95). Butzler, Skirrow, Blaser, and Campy-BAP selective agars were frequently found to produce plates with contaminant flora (e.g. coliforms, *Pseudomonas* spp., fecal streptococci, and yeasts) (92, 95), and studies using these media have reported poor or no recovery (32, 33, 94). Implicit in the use of selective media that incorporate antibiotics is that antibiotic-sensitive campylobacters may not be recovered.

The optimal incubation temperature and duration for the recovery of thermotolerant campylobacters on selective agar is reported as 42 °C for 48 h (92). Scoring the plates after 24 h may increase the likelihood of false-negative results, while increasing the incubation period to 72 h increases overgrowth by contaminants (e.g. *Proteus* sp. and *Bacillus* sp.) (92). In addition to the choice of plating medium, the failure to detect campylobacters in 600 waterfowl fecal samples, reported by Faere et al. (33), might also be in part explained by the Butzler agar plates being scored after only 24–28 h at 42 °C, perhaps leading to false-negative results.

An alternative approach to detecting *Campylobacter* spp. utilizes membrane filtration of a suspension of fecal or caecal samples or of enrichment broth cultures (18, 96, 97). After filtration the membranes are incubated on selective agar. When the membrane filtration method was compared to direct plating for the recovery of thermotolerant campylobacters from the caecal contents of ducks it was, however, found to be inferior (18). The suitability of membrane filtration in clinical investigations has also been evaluated. Le Roux and Lastovica (97) reported a higher recovery of a wide range of *Campylobacter* species after the filtration of diluted fecal samples and incubation of the filters on tryptose blood agar. The membrane filtration approach sometimes gives better recovery than direct plating and might be appropriate after negative results have been obtained by direct plating (98). In contrast, however, Kulkarni et al. (99) found it less sensitive than direct plating and not appropriate for routine clinical investigations.

The application of molecular-genomic methods (e.g. PCR) to the determination of campylobacters in the feces of waterfowl may provide a more accurate and reliable account of the incidence of *Campylobacter* spp. PCR also potentially reduces the time for detection and eliminates the need for conventional confirmatory methods, although standard PCR methods do not provide information about the viability of cells. Successful detection of naturally occurring *Campylobacter* species by direct PCR (i.e. without preliminary enrichment culture of samples), or by PCR after a selective enrichment step (e.g. M-Exeter broth, incubated at 37 °C for 4 h followed by 48 h at 42 °C), has been applied to duck, bovine, and poultry feces (24, 25, 100, 101). Comparative studies that evaluated PCR and conventional culture methods for the recovery of campylobacters from human fecal samples showed that PCR is superior. It shortens the time required for detection (8 h compared to 48–96 h by conventional methods). PCR also provides accurate identification of less common species (i.e. *C. upsaliensis* and *C. helveticus*) (102). In contrast, conventional culture techniques were found to be as good as PCR for the recovery of the most common species (i.e. *C. jejuni* and *C. coli*) from clinical stool samples but potentially missed uncommon species (99). A disadvantage of the PCR approach, however, is that it does not provide isolates for further diagnostics (e.g. sensitivity to antibiotics), thus conventional methods remain appropriate for the detection of thermotolerant campylobacters in clinical fecal samples (99). Problems found in the application of PCR to clinical stool samples also potentially apply to its use with

avian feces; further evaluation of PCR protocols for the detection of campylobacters in the feces of waterfowl is needed.

Detection in Aquatic Environments. The presence of stressed *Campylobacter* cells in small numbers, against a large background of native bacterial flora, necessitates the application of extremely sensitive recovery methods. The usual approach to the detection of campylobacters in aquatic environments involves their concentration, from large volumes of water, onto membrane filters and the incubation of these filters in a selective enrichment broth at 37 °C followed by incubation at 42 °C, and streaking of broth cultures onto selective agar followed by further incubation (103). Such procedures have been employed for the determination of campylobacters in diverse waters (104, 105). To increase sensitivity (i.e. increase the number of *Campylobacter* cells concentrated on the filter), the filtration of larger volumes of water samples seems appropriate (106). Thus Hänninen et al. (38) showed that the filtration of up to 10 L of drinking water increased the number of campylobacters recovered and suggested that sample volumes of 1000 mL are too small for the routine detection of campylobacters in drinking water. Similarly, the filtration of large sample volumes (1000–4000 mL) was reported for the determination of campylobacters in fresh and marine waters (50, 55). Pearson et al. (107) failed to isolate *Campylobacter* spp. by filtration of 10-mL samples of groundwater, whereas Stanley et al. (54) reported successful recovery of campylobacters from larger volumes (100–500 mL) of groundwater and recommended their use. However, the filtration of large volumes (1000 mL) of turbid surface water can lead to false-negative results. This is because the growth of high levels of background heterotrophs and coliforms during the enrichment stage prevents the growth of campylobacters to detectable levels, perhaps because of competition for nutrients (53).

An alternative method for the recovery of campylobacters from aquatic environments was proposed by Blaser and Cody (108). Their method required the filtration of water samples, followed by placing the filters face-down on *Campylobacter*-selective agar. The filters were removed after incubation at 42 °C for 12 h, and the plates were further incubated for another 48 h at 42 °C. The method was tested on seeded samples of sterile pure water and streamwater (108). Presumptive colonies were then subjected to confirmation. Successful determination of naturally occurring *Campylobacter* spp. in pond, river, and marine waters has been described using the incubation of membrane filters on selective agar (42, 48, 52); generally 0.45 µm filters were used, and incubation was 48 h at 42 °C. Blaser and Cody (108) found that incubating the filters face-down was more sensitive than face-up, so it is not clear why Arvanitidou et al. (42, 48) and Mawer (52) utilized the face-up approach. The incubation of filters on agar may, however, be unsuitable for testing turbid surface water. We compared the incubation of filters face-up on mCCDA selective agar with conventional incubation of filters in Preston enrichment broth for the recovery of campylobacters from turbid pond water in northeast England. The incubation of filters on mCCDA selective agar at 42 °C for 24–48 h gave no positives ($n = 12$ 100-mL samples), whereas 90% of samples gave confirmed *Campylobacter* isolates after selective culture of membrane filters in Preston broth, incubated at 37 °C for 24 h and 42 °C for 24 h followed by plating on mCCDA selective agar and incubation at 37 °C for 48 h (unpublished data). Similarly, Rosef et al. (45) found that enrichment broth culture of membrane filters (in Preston broth incubated at 42 °C for 48 h) gave greater recovery rates of campylobacters from river waters than direct incubation of filters on mCCDA selective agar incubated at 42 °C for 24–48 h.

In general, the enrichment step aims to resuscitate damaged cells. Thus the enrichment culture of membrane filters in basal or selective broth significantly increased the recovery of experimentally injured campylobacters in water (109). An enrichment regime that involved a 4-h incubation in broth culture (nutrient broth containing 5% lysed horse blood, 0.02% sodium metabisulphite, 0.02% sodium pyruvate, and 0.05% ferrous sulfate) at 37 °C, prior to incubation for 44 h at 43 °C, gave improved recovery of damaged *Campylobacter* cells in river water (110), perhaps by allowing repair of sublethally injured cells prior to their exposure to higher temperature (110, 111). The importance of an enrichment step in the determination of *Campylobacter* spp. in natural waters is well documented (39, 45, 54, 112), although with heavily contaminated samples (e.g. sewage) the enrichment step may have no beneficial effect on recovery rate, and indeed may be counterproductive due to competition from other microflora (112). Standardization of solid/liquid media for the detection of campylobacters in aquatic environments is not yet achieved. Nevertheless, the combination of Preston broth for selective enrichment and mCCDA for selective plating has been recommended for the detection of *Campylobacter* spp. in potable and environmental water samples where the organism occurs in relatively low numbers (96).

Confirmation of *Campylobacter* isolates is routinely based on morphological (microscopic) features and biochemical (oxidase and catalase positive) tests and on growth under microaerobic but not aerobic conditions (113). These tests are largely limited to the confirmation of presumptive isolates to genus level, and they may generate false-positive results. PCR can reinforce the conventional confirmation of presumptive isolates and also provide further information about the species recovered, if species-specific primers are used (53). The value of PCR as a confirmatory test was shown by Diergaardt et al. (114) who isolated 100 presumptive campylobacters from drinking water, groundwater, and raw sewage in South Africa. Of these isolates, 22 grew under microaerobic but not aerobic conditions and were thus confirmed as *Campylobacter* spp. by biochemical tests. PCR followed by the analysis of a 16S rRNA sequence, however, showed that only three of the 22 confirmed isolates were actually *Campylobacter jejuni*, while the remaining 19 isolates were shown to be *Arcobacter butzleri*.

Various PCR protocols, using diverse primers, have been employed to detect campylobacters in water and wastewater (37, 115–118). Most detected the presence or absence of campylobacters; others (119) used real-time PCR with the aim of obtaining quantitative results. These protocols were applied to the recovery of *Campylobacter* spp. from samples that had been liberally seeded with *Campylobacter* cultures. Also, direct PCR assay for the detection of naturally occurring campylobacters in contaminated drinking water and swimming pool water has been successful without the need for enrichment culture (120, 121). Furthermore, Yang et al. (119) suggested that they had detected *C. jejuni* in naturally contaminated environmental water by direct PCR without the need for prior enrichment culture of the samples. The description of methods in their paper implies, however, that there was enrichment culture prior to PCR. Thus, although direct detection of *Campylobacter* spp. by PCR in clean water (e.g. drinking water) may be feasible, the direct application of PCR to turbid environmental samples (e.g. pond water) poses problems and may give false negative results (26). Campylobacters in turbid environmental waters are liable to be found in relatively low numbers which, unlike in contaminated drinking water or swimming pool water, are present against an abundant background microflora and high concentration of potential PCR inhibitors.

PCR assay combined with prior selective enrichment increases the effectiveness of PCR detection of campylo-

TABLE 3. Molecular Methods Used To Assess Clonal Diversity of *Campylobacter jejuni* in Waterfowl and Other Avian Species

sample location	sample source ^a	detection methods	molecular confirmation	target genes	refs
East Yorkshire, U.K.	mallard	enrichment and selective plating	PCR	<i>flaA/flab</i>	26
Germany, France, The Netherlands	poultry	enrichment	PFGE, PCR-RFLP	<i>smal; fla</i>	148
Sweden	migratory birds ^b	selective plating	multiplex PCR; PFGE	23S rRNA; <i>smal</i>	138
Lancashire, U.K.	poultry	enrichment	MLST	<i>aspA; glnA; gltA; glyA</i>	143
Cheshire, U.K.	poultry	enrichment and selective plating	MLST	<i>aspA; glyA</i>	145
U.K., Denmark, South Africa, The Netherlands, Sweden, France	poultry	selective plating	PCR-RFLP	<i>flaA/flab</i>	149

^a Feces. ^b Jackdaw, starling, dunlin, white wagtail, long-eared owl, blackbird, song thrush, sparrowhawk.

bacters in environmental waters by increasing the number of target cells. This was noted with spiked environmental waters (37, 117, 122) and with naturally occurring cells in environmental waters and sewage (24, 26, 37, 117, 123, 124). Thus, minimum detection limits of 3, 13, and 30 cells per 100 mL were obtained for river (117), wastewater (117), and estuarine water (122), respectively, using enrichment of seeded samples followed by PCR. This is better than the 400 cells per 100 mL required for direct detection by PCR in turbid pond water (26).

A potential disadvantage in the application of direct PCR to environmental samples, without an enrichment step, is the possibility that fragments of naked DNA or DNA in dead cells might be detected (125). Dead *Campylobacter* cells in environmental waters suggest that the water has been contaminated, but no longer poses any threat to public health (126). Since the detection of viable *Campylobacter* cells is the relevant issue, the application of an enrichment step prior to PCR assay, encouraging the detection of viable cells only, is a sensible approach. PCR assay after selective enrichment is potentially a standard method for the detection of presence or absence of campylobacters in environmental samples (26, 124). An alternative approach to distinguishing whole cells from DNA fragments is fluorescence in situ hybridization (FISH). Whole cells are labeled using a fluorescent *Campylobacter*-specific oligonucleotide probe and are then observed under an epifluorescence microscope. Lehtola et al. (127) were able to detect *C. coli* following membrane filtration of tap water spiked with *C. coli*; they also found that difference in the fluorescence brightness of hybridized cells allowed separation of actively growing and senescent *Campylobacter* cells.

Typing of *Campylobacter* from Waterfowl and Aquatic Environments. The typing of confirmed *Campylobacter* isolates can provide important epidemiological information relevant to (i) tracking the route of transmission to humans; (ii) monitoring the geographic and temporal distribution of specific strains; and (iii) developing control strategies (128). Methods include serotyping, phage typing, phenotyping, and more recently developed molecular-genomic approaches.

Serotyping methods, based on the agglutination of specific antigens with antisera (129–131), are often used in clinical settings, but they have the problem that many strains of confirmed *Campylobacter* isolates from human and environmental sources cannot be typed (41, 52, 132, 133). Phage typing (134, 135) may be used in combination with serotyping and can help with this problem (128). Phenotyping methods are usually based on biochemical tests (hippurate hydrolysis, H₂S production) and may be valuable for differentiating

between thermotolerant campylobacters, particularly *C. jejuni* and *C. coli* (136), but are limited in their ability to discriminate between many members of the family Campylobacteriaceae (137).

DNA-based typing methods are potentially more sensitive than conventional ones. Examples of their application to typing of *C. jejuni* of avian origin are given in Table 3. The methods include the following: digestion of bacterial DNA using restriction enzymes followed by pulsed-field gel electrophoresis (PFGE) (138); random amplification of polymorphic DNA (RAPD) (139); amplified fragment length polymorphism fingerprinting (AFLP) (140); polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (141); flagellin typing (*fla* gene) (142); and direct nucleotide sequencing (with or without prior PCR) (26, 142–144). Recently, the application of multilocus sequence typing (MLST) to the differentiation of strains of *C. jejuni* and *C. coli* has provided sensitive results and is increasingly recognized as an accurate typing tool for the differentiation of *Campylobacter* spp (143, 145–147).

Such methods have suggested that genetic instability occurs in *Campylobacter* strains recovered from poultry (148), but this is questioned by other studies. For example, analysis of PFGE profiles of *Campylobacter* isolated from migratory birds in Sweden showed that isolates from one bird species, or feeding guild, often exhibited high similarity, indicating a common transmission source for groups of individuals, or an association between particular subtypes of *Campylobacter* spp. and particular phylogenetic groups of birds (138). Similar observations were made by Colles et al. (143) and French et al. (145) using MLST on isolates from starlings and poultry, respectively, in northwest England and by Abulreesh (26) using DNA sequencing of PCR products of isolates from mallard ducks in northeast England. This evidence of high genetic similarity of *Campylobacter* species associated with particular bird species may also reflect host adaptation (143) and overall genetic stability of *Campylobacter* strains associated with particular bird species (149). It is important, however, to be aware that the comparison of data from these studies is not straightforward because of the different molecular methods applied.

Although DNA-based methods are powerful tools the routine typing of campylobacters generally still relies on serotypic or phenotypic methods. This is largely because the DNA-based methods require more expensive equipment and reagents, and the preparation of gels is time-consuming and tedious (128). Another disadvantage in the application of DNA-based methods (e.g. *fla* gene typing) is the use of diverse protocols that hinders comparison between different studies

(128). It is generally accepted that one ideal typing procedure is not available, indeed the application of both conventional and molecular techniques in a synergistic manner may enhance the reliability of results (37, 136, 150). Collaboration between laboratories to standardize typing techniques may lead to a profile database for typed isolates that will enable the monitoring of trends in *Campylobacter* infections worldwide (128).

Concern about avian influenza has raised awareness of the potential for long-distance carriage of disease by migratory birds (9). Whether waterfowl have a role in the dissemination of *Campylobacter* spp. that results in increased human disease is likely to be elucidated through development and greater use of typing methods. Typing might allow links to be established between isolates of avian, environmental, and human origin. This is important in the context of *Campylobacter* infections in humans potentially extending beyond gastroenteritis; there has been, for example, realization that some forms of the neuromuscular disorder Guillain-Barré syndrome are associated with prior *C. jejuni* infection (151).

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