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The speciation and subtyping of campylobacter isolates from sewage plants and waste water from a connected poultry abattoir using molecular techniques

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SUMMARY

In this study the distribution of phenotypes of campylobacter strains in sewage and surface waters was investigated by subtyping and by speciation of isolates from various aquatic environments. These environments included two municipal sewage plants (SPA and SPB) and waste water from a poultry abattoir (WWA). Both the sewage plants SPA and SPB collected domestic and industrial waste, and SPA received drain water from WWA. SPB received no waste water from any meat-processing plant. The isolates were speciated by PCR and subtyped by PCR/RFLP based on the flagellin PCR products.

From all three reservoirs, no *Campylobacter lari* was isolated, and approximately 80% of the isolates could be identified as *C. jejuni* and the rest belonged to the *C. coli* species. The PCR/RFLP typing technique has a high discrimination level and was reproducible between two separate laboratories. The 182 isolates tested yielded 22 distinct *Dde* I profiles. The results indicate that strains with profiles found in poultry are also detectable in waste water presumed to be solely from domestic and human sources. In addition some strains were unique to the known poultry-related sources, suggesting that avian-specific strains, non-pathogenic to man, may exist in the environment. In contrast some strains were unique to human waste indicating the potential importance of non-poultry sources of infection. No seasonality was observed in the profile distribution. So, at least in the Netherlands, it is unlikely that infections caused by contaminated surface waters contribute to the seasonality of human campylobacteriosis.

INTRODUCTION

In developed countries *Campylobacter jejuni* is a common cause of acute bacterial enteritis in man [1]. In the Netherlands it has been estimated that the

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annual incidence of campylobacteriosis is 1200–2500 cases per 100000 [2]. The associated economic consequences in terms of lost working days and cost of treatment are considerable.

Undercooked poultry meat is generally regarded as the predominant source of infection. However, campylobacters are ubiquitous in the environment and exposure to untreated water is another recognised source [3]. Campylobacters can survive well in aquatic environments, especially when temperatures are low [4]. Faeces from asymptomatic, wild and domestic animals, like cattle, swine, poultry, waterfowl, rodents and dogs, all contribute to contamination of surface waters [5, 6]. In particular poultry abattoir effluent can contain high numbers of campylobacters [7–9].

The contribution of environmental campylobacters as a source of human and animal campylobacteriosis is currently unknown. However, previous environmental surveys indicate a seasonal variation of campylobacters [8] exemplified by a decline in numbers of organisms observed in the sewage of an activated sludge system during the end of the summer [9]. Even though this seasonality contradicts that often observed in human infections of the developed world [1] the possibility cannot be excluded that campylobacters with enhanced virulence properties are better able to survive aquatic environmental conditions during the summer months. Such anomalies need to be investigated further. Accurate methods for speciation and subtyping are particularly important in such studies.

Until recently campylobacter speciation has been difficult. Discrimination between closely related thermophilic species, *C. jejuni*, *C. coli*, and *C. lari*, on the basis of phenotypic characteristics is time consuming and largely inaccurate [10–12]. Recently, a polymerase chain reaction (PCR) technique based on species-specific 23S rRNA fragments has been developed which may be suitable for rapid speciation of campylobacter strains [13].

Techniques for campylobacter subtyping have in the past caused even more difficulties. Conventional subtyping methods, such as biotyping [14, 15] and serotyping [16, 17] are not readily available and have poor levels of discrimination. Recently techniques developed for the restriction fragment length polymorphism (RFLP) analysis of the flagellin genes [18] appeared to provide a suitable and practical typing tool [19].

The aim of the current study was to utilize these newly developed epidemiological tools to speciate and subtype campylobacters isolated from several water sources. These sources have previously been described and include poultry abattoir effluent and an associated as well as an unrelated sewage works sampled over a 1-year period [9].

MATERIALS AND METHODS

Bacterial strains

Isolates from the influx and efflux of two municipal plants were included in this study [9]; namely an activated sludge sewage plant (SPA) and a trickling filter sewage plant (SPB). The activated sludge system, with a capacity of 60000 citizen equivalents, receives sewage from households and from various small industries,

including a poultry abattoir. The trickling filter system, with a capacity of 130000 citizen equivalents, receives domestic and industrial waste, but not from meat-processing industries. Furthermore, isolates obtained from the waste water of the poultry abattoir (WWA), connected with the activated sludge plant, were also characterized. The poultry abattoir is located at a distance of approximately 4 km from the activated sludge system. Samples were collected from all three reservoirs over a 1-year period.

The samples were cultured by selective enrichment (42 °C) in Charcoal Cefoperazone Deoxycholate Broth, after a pre-enrichment step (37 °C) according to previously published methods [9]. Confirmation of suspected *Campylobacter* isolates was based on microscopic appearance, growth under micro-aerobic conditions in Brain Heart Infusion Broth (BHI, 0037-01-6 Difco Laboratories, Detroit, USA) at 42 and 25 °C, oxidase and catalase activity.

The isolates were stored at −80 °C in BHI containing 20% (v/v) glycerol. For DNA-preparations, isolates were cultured in BHI for 2 days at 42 °C under micro-aerobic conditions.

DNA preparations

A BHI-culture was swabbed onto Columbia Agar Base plates (CM 331 Oxoid, Basingstoke, UK) with 5% (v/v) defibrinated, lysed horse blood (CAB) and incubated for 2 days at 42 °C under micro-aerobic conditions. Bacteria were harvested and washed in neutralized bacteriological peptone (1 g l^{-1}, L34 Oxoid) containing 8·5 g l^{-1} NaCl and finally resuspended in 100 μl lysis buffer of the IsoQuick® Nucleic Acid kit (MXT-020-100, MicroProbe Corporation, Washington, USA). The nucleic acid extraction and purification procedure was carried out according to the manufacturer's instructions. The extraction is based on the chaotropic properties of guanidine isothiocyanate, which both disrupts cellular integrity and inhibits nuclease (DNAase and RNAase) activities, thereby providing both cell lysis and DNA stabilization. The pure nucleic acids were dissolved in 100 μl RNAase free water and stored at −20 °C.

Speciation

The species were discriminated by PCR, based on the 23S rRNA gene [13]. Briefly, a standard PCR reaction mixture (50 μl) contained 5 μl of the DNA solution and 45 μl of buffer to give final concentrations of 20 mM Tris-HCl (pH 8·8), 50 mM-KCl, 3 mM-$MgCl_2$, 0·01% (w/v) gelatin, 200 μM of each deoxyribonucleotide triphosphate (u1240 Promoga Corporation, Madison, USA), 1 U of AmpliTaq DNA polymerase (N808-1012, Perkin-Elmer Cetus, Norwalk, USA) and 50 pmol of each primer. The reaction mixture was overlayed with 50 μl mineral oil (M3516 Sigma) to prevent evaporation. Amplification was carried out using thermocycler (Perkin-Elmer 480) with the following profile for 27 cycles: 1 min at 94 °C; 1 min at 54 °C and 1 min at 74 °C. Amplified samples were separated by agarose gel electrophoresis (1·5% (w/v) 1444964 Agarose MP, Boehringer Mannheim Biochemica) in a TAE buffer (40 mM (Tris pH 7·2) – 20 mM acetic acid – 50 mM EDTA) and visualized by ethidium bromide staining (0·5 μg/ml) [20].

Typing by PCR/RFLP

PCR: Samples of the extracted DNA were assayed in a PCR/RFLP system, as described by Ayling and colleagues [18]. A standard PCR reaction mixture (50 μl) was composed of Saiki buffer (end concentration 3·0 mM $MgCl_2$, 1% (w/v) gelatin, 10 mM Tris-HCl pH 8·5, 50 mM KCl), 5% (v/v) glycerol, 500 μM of each deoxyribonucleotide triphosphate (Promega), 2·5 U of AmpliTaq DNA polymerase (Perkin–Elmer Cetus) and 50 pmol of each primer and contained 2 μl extracted DNA solution. This solution was overlayed with 50 μl mineral oil to prevent evaporation. The amplification profile started with a denaturation step (94 °C; 60 s) followed by 45 cycles of amplification in a thermocycler (Perkin–Elmer 480). Temperature profile comprised 45 s at 94 °C; 45 s at 55 °C and 2 min at 72 °C. The programme was terminated by an extension step (58 °C; 90 s) and an incubation step (72 °C; 5 min). Primers, based on the flagellin genes, used were: Cj431: 5′-AAAGGATCCGCGTATTAACACAAATGTTGCAGC-3′, Cj432: 5′-AAAGGATCCGAGGATAAACACCAACATCGGT-3′ and Cj433: 5′-GATTTGTT-ATAGCAGTTTCTGCTATATCC-3′ [18].

Digestion: The PCR product was digested by incubating 12 μl PCR mixture with 10 U. *Dde* I restriction enzyme (15238-025 Gibco BRL, Life Technologies Ltd, Gaithersburg, USA), 2 μl 10 × buffer containing 500 mM Tris-HCl (pH 8·0), 100 mM–$MgCl_2$, 500 mM-NaCl (Life Technologies Ltd) and 5 μl Millipore water for 2 h at 37 °C. Two microlitres of loading buffer (40% (w/v) sucrose in water containing 0·25% (w/v) bromophenol blue) were added to the digestion mixture to stop the digestion reaction. The digestion product was separated on a 2·5% (w/v) agarose gel (2·0% (w/v) NuSieve® GTG® Agarose (50082, FMC, Rockland, UK), 0·5% (w/v) MP agarose (Boehringer Mannheim Biochemica)) in TAE buffer. The fragments were visualized by staining with ethidium bromide (0·5 μg/ml) [20]. Profiles were compared by using the computerized Logitech Foto Touch scanning system and analysed by the Singularity RFLP analyzer 1.21 (Ultraviolet Products Ltd, Cambridge, UK).

RESULTS

Campylobacter isolates ($n = 165$) from the three different reservoirs (WWA, SPA and SPB) and collected over a 1-year period were identified at the species level using the PCR technique with primers specific for *C. jejuni*, *C. coli*, and *C. lari*. Approximately 10% of strains investigated could not be speciated by this technique. All the isolates identified as *C. jejuni* gave PCR products of 710 or 810 bp. The size of this PCR product was independent of the reservoir and of the season. The species distribution was similar for the three reservoirs investigated (Table 1). No *C. lari* strains were identified in isolates from any of the reservoirs.

For PCR/RFLP the bacterial culture, DNA extractions, PCR's and digestions were performed in duplicate for each strain. Analysing standard strains and comparing the results between two independent laboratories (CVL [UK] and WAU [the Netherlands]) demonstrated the consistency of this typing method. The 1·49 kb PCR products from the flagellin genes of each of 182 isolates were digested with *Dde* I to yield a diversity of profiles each comprising 2–5 fragments

Table 1. *The distribution of the species among the isolates from reservoirs WWA, SPA, and SPB*

Reservoir	Number	Number of isolates	
		C. jejuni (%)	*C. coli* (%)
WWA†	78	56 (72)	16 (21)
SPA	60	44 (73)	11 (18)
SPB	44	30 (68)	8 (18)

* Percentage of total isolates (n = 165), which could be identified as specific species.

† WWA, poultry abattoir waste water, which is drained on the activated sludge system (SPA). SPA, municipal sewage plant; an activated sludge system. SPB, municipal sewage plant; a trickling filter system.

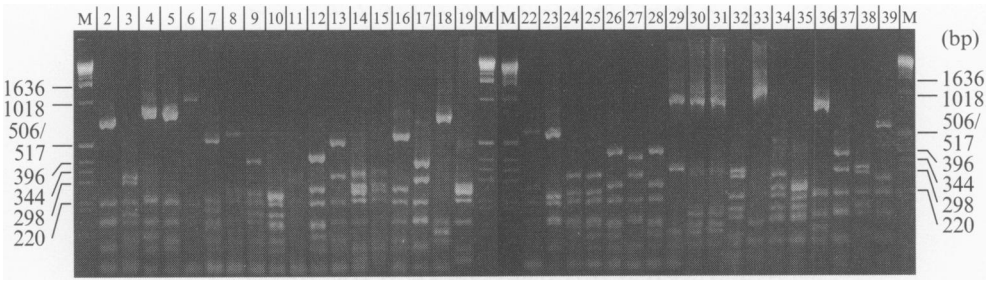

Fig. 1. Gel electrophoresis of individual PCR/RFLP profiles from campylobacter isolates originating from sewage plants (SPA and SPB) and poultry abattoir waste water (WWA). Lanes 1, 20, 21, 40: molecular weight markers. Fragment sizes are shown beside the lane. Lanes 2–19, 22–39: designated profiles; all isolates are shown as numbered in Table 2.

sized between 124 bp and 1075 bp (Fig. 1). Among the isolates tested 22 distinct *Dde* I profiles were distinguished. None of the 22 profiles matched with previously described 13 profiles [18] and therefore the 22 profiles observed in this investigation was designated a profile number extending the previous scheme (Table 2). Approximately 52% (95/182) of the strains fell into only four profiles (profiles 14, 15, 22, and 23). Hunter [21] suggested that discriminatory power can be defined mathematically as the probability that two strains chosen at random from a population of unrelated strains will be distinguished by that typing method. The numerical index of discriminatory power for the PCR/RFLP typing method, defined using Hunter's technique, was 0·92.

The distribution of the profiles among the isolates from the three reservoirs is illustrated in Fig. 2. The ranked order of prevalence of the various profiles was dependent on the source. Only six profiles (profiles 14, 22, 15, 23, 32, and 19) were represented among isolates from each of the three reservoirs. Some profiles were unique to isolates from WWA (profiles 25, 28, 21, and 29), SPB (profiles 31, 33, and 34) or SPA (profile 35). It is notable that no profiles were confined to WWA and SPB only.

Samples were collected from SPA, SPB and WWA over a 1-year period. Strains expressing multiple profiles were isolated from 23 out of 63 samples (Table 3). There was no evident seasonality in any of the strains isolated from any of these reservoirs.

Table 2. *Details of campylobacter strains used for PCR/RFLP experiments*

Lane no. in Fig. 1	Isolate	Reservoir	Date of isolation	PCR/RFLP profile	Lane no. in Fig. 1	Isolate	Reservoir	Date of isolation	PCR/RFLP profile
2	WAU705*	SPA†	05.08.92	14‡	22	WAU1119	WWA	10.03.93	17
3	WAU1000	SPB	27.01.93	31	23	WAU1280	WWA	21.04.93	16
4	WAU905	SPA	09.12.92	15	24	WAU1120	WWA	10.03.93	27
5	WAU1053	SPA	12.02.93	15	25	WAU1159	WWA	26.03.93	27
6	WAU721	SPA	05.08.92	34	26	WAU1160	WWA	26.03.93	28
7	WAU719	SPA	05.08.92	16	27	WAU1162	WWA	26.03.93	25
8	WAU761	SPA	26.08.92	17	28	WAU1182	WWA	28.03.93	28
9	WAU724	SPA	05.08.92	18	29	WAU1184	WWA	28.03.93	29
10	WAU731	WWA	05.08.92	19	30	WAU1269	SPA	21.04.93	30
11	WAU1229	WWA	31.03.93	20	31	WAU631	SPB	24.06.92	30
12	WAU738	WWA	05.08.92	21	32	WAU671	SPB	15.07.92	31
13	WAU754	WWA	26.08.92	22	33	WAU527	SPB	01.04.92	32
14	WAU763	WWA	26.08.92	23	34	WAU526	SPB	01.04.92	33
15	WAU1155	SPA	26.03.93	23	35	WAU522	SPB	01.04.92	19
16	WAU810	SPA	21.10.92	24	36	WAU929	SPB	09.12.92	15
17	WAU824	WWA	21.10.92	25	37	WAU1067	WWA	22.02.93	25
18	WAU1161	WWA	10.03.93	26	38	WAU1216	SPB	31.03.93	31
19	WAU1212	SPB	31.03.93	35	39	WAU1116	WWA	10.03.93	24

* WAU, Wageningen Agricultural University, The Netherlands.
† For abbreviations, see Table 1.
‡ For profiles, see Fig. 2.

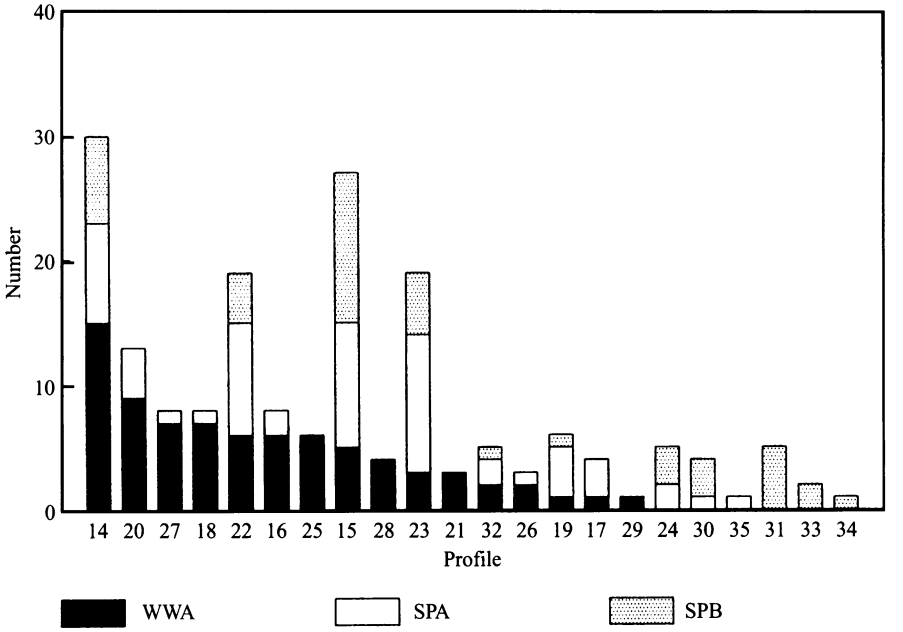

Fig. 2. Distribution of PCR/RFLP profiles among 182 campylobacter isolates obtained from sewage plants (SPA and SPB) and poultry abattoir waste water (WWA), with the profiles on the X axis according to decreasing order of prevalence among the waste water isolates.

Table 3. *The number of isolates and profiles observed in samples taken over a 1-year period*

Number of samples with no. of multiple isolates	Number of observed profiles				
	1	2	3	4	5
1	24	0	0	0	0
2	7	6	0	0	0
3	1	2	6	0	0
4	0	1	1	1	0
5	2	0	2	2	0
6	1	1	0	1	0
7	0	0	1	0	0
8	0	1	0	2	0
10	0	0	0	0	1

DISCUSSION

In this study the distribution and diversity of campylobacters in the influx and efflux from two municipal sewage plants in the Netherlands was investigated. The sewage plants differed in their purification mechanisms; one being an activated sludge system (SPA), the other a trickling filter system (SPB). In addition isolates were obtained from the waste water (WWA) of a poultry abattoir which drained into SPA. Both sewage plants received waste from households and various small industries. However, only SPA received waste from a meat processing plant. The isolates were characterized both at the species level, by species-specific PCR [13] and at the subtype level, by PCR/RFLP profile based on the flagellin genes [18].

The molecular technique used for speciation [13] was very easy to perform. Unfortunately, 10% of the campylobacter strains did not result in a PCR product with the available species-specific primers [13]. The proportion of unspeciated strains was equally distributed between the three sources. No explanation is currently available for the inability to speciate these anomalous strains, using these primers. However these strains had phenotypic characteristics of *C. jejuni/coli*. An additional multiprimer PCR, including primer sets of several species [22], was found to be unsuitable because of non-specific amplification (data not shown). The species distribution was independent of the reservoir investigated; about 80% of isolated strains were *C. jejuni*, 20% were *C. coli* and *C. lari* was not found. These results confirm previous studies, using phenotypic techniques [23]. However, in other investigations [6] the proportion of *C. coli* isolates was significantly higher (66%) and *C. lari* was also recoverable. The reasons for these differences are unknown but may reflect differences between species in sensitivity to selective antibiotics [24, 25] and survival in aquatic environments [26].

The PCR/RFLP technique has only recently been developed [18, 27, 28] and its value in the epidemiological investigation of campylobacters is still being established [18]. Nevertheless the evidence to date suggests that there are a number of advantages over alternative typing methods. In particular the technique is readily available, has a high level of discrimination and provides comparable results between laboratories. Moreover, non-typable isolates were not obtained.

From the 182 isolates investigated 22 PCR/RFLP profiles were distinguishable. More than half (52%) of the isolates were confined to four profiles (profiles 14, 15, 22, and 23) suggesting that certain strains either have enhanced abilities to colonize host sources or have enhanced survival potentials in the environment. However, the absence of profile variation with season would suggest that environmental susceptibility is not a factor in this strain distribution.

There were marked differences in the prevalence of profiles among the isolates from the various reservoirs. Strains of six profiles were common to all three sources indicating that those strains which were present in poultry can also occur in waste from human sources. Strains of a further ten profiles were either confined to both WWA and SPA or unique to WWA only. This is consistent with the likelihood that strains from poultry abattoir, WWA, would be represented in the receiving sewage plant, SPA. However, as these profiles are not observed in strains from SPB, this may suggest that at least some strains may be confined to poultry only and may not occur in human domestic waste water. Previous experimental evidence using *in vitro* and *in vivo* models has indicated the presence of some campylobacter strains in the environment with reduced virulence [29] and which may not, therefore, be pathogenic to man. In contrast, strains of the remaining six profiles were from SPA and/or SPB only. Thus no profiles were observed which were confined to strains from SPB and WWA. Therefore, it is possible that the source of some campylobacter strains infecting humans, and hence present in human sewage effluent, is not poultry. The future subtyping of human faecal isolates by PCR/RFLP may enable clarification of this situation.

Interestingly all of the isolates from the poultry abattoir effluent waste had different profiles from the 13 profiles previously observed in poultry in the UK

[18]. Whether this discrepancy is a true reflection of the geographical variation in campylobacter strains present in the Netherlands and UK has yet to be determined by the investigation of larger numbers of samples and comparable isolates from similar sources.

The absence of seasonality in the profile distribution is consistent with previous observations relative to the prevalence in surface water, sewage and broilers [9, 30]. Moreover, total campylobacter numbers were previously shown to decline in the summer months [8]. It, therefore, seems unlikely, at least in the Netherlands, that infections caused by contaminated surface waters contribute to seasonality of human infections.

Despite these, and other investigations, the sources of the majority of sporadic cases of human campylobacteriosis remain unproven. In the past such studies have been limited by the availability of adequate subtyping techniques. This study has utilized a recently developed molecular typing scheme to investigate the potential health risk of surface waters contaminated with campylobacters. However, not all environmental campylobacters may be pathogenic for man. In addition the results suggest that although poultry may have an important role in human infections, other sources cannot be ignored. The introduction of such molecular epidemiological tools will enable routes of transmission of campylobacter infections to be definitively established in the future.

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