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Distribution of *Campylobacter jejuni* Penner serotypes in broiler flocks 1998–2000 in a small Danish community with special reference to serotype 4-complex

A. WEDDERKOPP[1], E. M. NIELSEN[2] AND K. PEDERSEN[1]*

[1] *Danish Veterinary Institute, Hangøvej 2, DK-8200 Aarhus N, Denmark*
[2] *Danish Veterinary Institute, Bülowsvej 27, 1790 Copenhagen V, Denmark*

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SUMMARY

During the period January 1998–December 2001, all Danish broiler flocks were monitored bacteriologically for thermophilic campylobacters and isolates were stored at −80 °C. Six neighbouring broiler farms in a small community were selected for detailed examination of all *Campylobacter jejuni* isolated ($n = 180$) from these farms during 1998–2000 using Penner serotyping and pulsed-field gel electrophoresis (PFGE). The area and the farms were selected according to their prevalence of campylobacter so that both farms with low and high frequencies of campylobacter positive flocks were included in the study. The frequency of campylobacter positive flocks on the six farms ranged from 24·5 to 72·7%. One hundred and eighty of the isolates were *C. jejuni* (included in this study), 14 isolates were *C. coli* whereas 7 isolates belonged to other species but were not further identified. By serotyping of all *C. jejuni* 56 isolates (31·5%) were assigned to the 4-complex, 32 isolates (18·0%) to serotype 2, 12 isolates (6·7%) to serotype 11, and 11 isolates (6·2%) were assigned to serotype 12. In three farms, 4-complex was the most prevalent serotype, in one farm it was the second most frequently isolated serotype, while serotypes 2 and 1,44, respectively, were the most frequently isolated from the two remaining farms. This serotype distribution differed from the overall country-wide distribution where serotypes 2 and 1,44 are the most prevalent. All serotype 4-complex isolates from the six selected farms were compared by PFGE to serotype 4-complex isolates from the rest of the country. The results showed that there was a high level of diversity among isolates from the whole country, whereas isolates from the six farms were very homogeneous and only displayed one or a few different PFGE patterns on each farm. It is suggested that certain campylobacter clones persist in a confined geographical area, probably at the farm, and that the broiler houses may be repeatedly infected with a few *C. jejuni* clones during succeeding broiler flocks. New clones may be introduced, however, the sources and vehicles are yet unknown.

INTRODUCTION

Campylobacter is worldwide one of the most important causes of bacterial gastroenteritis, and in many countries, campylobacter has outnumbered other bacterial intestinal pathogens [1–3]. In many industrialized countries, the number of human cases of campylobacter infections has increased dramatically in the recent years, and so far, no one has been able to explain why. *Campylobacter* spp. are widespread in nature, wildlife and livestock. Poultry has in particular been incriminated as an important reservoir for human campylobacteriosis [2, 4, 5], and in a recent Danish case-control study, consumption of undercooked

* Author for correspondence: Danish Veterinary Institute, Department of Poultry, Fish and Fur Animals, Hangøvej 2, DK-8200 Aarhus N, Denmark.

Fig. 1. Map of Denmark showing the positions of farms with *C. jejuni* 4-complex isolates. The figures correspond to 20 different PFGE patterns found throughout the country. Farms marked ● have PFGE pattern 7 ($n=7$). At farms marked ◎ the PFGE pattern was found in two farms. The rest of the farms are marked ○ indicating that the PFGE pattern was found only once. ■ indicates the six farms located in the small community under study.

poultry was identified as an important risk factor [6]. During the period January 1998–December 2001, all Danish broiler flocks were monitored for campylobacter colonization at our laboratory [7]. During the 3 years 1998, 1999 and 2000 a total number of 18 168 poultry batches were slaughtered, an average of 42·5% being positive for *Campylobacter* spp.

Several genotyping and phenotyping methods have been applied to *Campylobacter* spp., e.g. serotyping [8, 9], phage typing [10], ribotyping, pulsed-field gel electrophoresis (PFGE), fla-typing [11], and amplified fragment length polymorphism (AFLP) [12]. AFLP and PFGE are both excellent techniques for discriminating between isolates, but only PFGE has been subjected to a standardization of protocols. Serotyping is so far the only definitive typing method, although its discriminatory power is limited [11]. In the present study, we therefore chose PFGE and serotyping as typing methods.

A Danish study using PFGE showed that certain clones of *C. jejuni* persisted during successive broiler flock rotations [13]. In order to study whether the clones of *C. jejuni* within a close neighbourhood were epidemiologically related, *C. jejuni* isolated from broilers on six farms in a local geographical area were studied more closely regardless of the frequency of campylobacter colonization. In particular, isolates belonging to the serotype 4-complex were compared with 4-complex isolates from the rest of the country.

METHODS

Campylobacter isolates and culture methods

The campylobacter isolates were collected during a 3-year period through the national campylobacter surveillance programme as previously described [7] and stored at −80 °C. A local geographical area in Jutland (Fig. 1) with a long tradition for broiler production was selected. In this area, the farm density was relatively high, and campylobacter occurrence included both low and high frequencies of positive

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Table 1. *Isolation of campylobacters from broilers on six farms in a small geographic area of Jutland during 1998–2000*

Farm	No. of samples	% campylobacter positive samples	*C. jejuni*	*C. coli*	*Campylo-bacter* spp.
A	54	81·5	40	2	2
B	40	57·5	18	3	2
C	102	67·6	64	4	1
D	80	16·3	13	0	0
E	68	32·4	19	3	0
F	102	29·4	26	2	2
Total	446	45·1	180	14	7

flocks. Six neighbouring broiler farms located within the area were selected for further investigation, whereafter all *C. jejuni* from these farms isolated during 1998–2000 were recovered for further investigation.

Bacterial isolates were cultured on modified CCDA (mCCDA) plates (blood-free agar base with cefoperazone, 32 mg/l and amphotericin B, 10 mg/l; Oxoid CM 739/SR 155). The plates were incubated for 48 h in a microaerobic atmosphere (6% CO_2, 6% O_2, and 4% H_2). During the period where the isolates were collected, the routine culture procedure was changed, so that during 1998, the plates were incubated at 42 °C, whereas in 1999 and 2000 they were incubated at 37 °C.

Serotyping

All *C. jejuni* isolates were serotyped according to the Penner serotyping scheme for heat-stable antigens by the use of passive haemagglutination in microtiter plates as previously described [9, 14]. Three dilutions of each antiserum were used (1:80, 1:640, 1:5120), and a clear reaction in the highest dilution was considered positive. Antisera were produced against all 66 serotype reference strains of the serotyping system [14, 15].

Pulsed-field gel electrophoresis

Isolates belonging to the serotype 4-complex were subjected to PFGE. There were 56 isolates from the six selected farms that were compared to 29 4-complex isolates from the rest of the country. PFGE was carried out using the protocol recommended by CAMPYNET (protocol by On SLW, Hänninen M-L, Thomson-Carter F, available from http://campynet.vetinst.dk/PFGE.html) and with *Sma*I as restriction enzyme. PFGE patterns were assigned arbitrary numbers 1–26.

Calculation of diversity

The diversity within each of the two groups of 4-complex isolates was calculated as described by Kühn et al. [16]. The PFGE patterns of all isolates were compared pairwise, yielding a total of $N_c = N \times (N-1)/2$ comparisons per group, where N is the number of isolates in each group. The number of comparisons yielding the same pattern was counted as N_1. From this a diversity index was calculated: $D_i = 1 - N_1/N_c$. Thus, a $D_i = 1$ would indicate that all isolates had different patterns, whereas a $D_i = 0$ would indicate that all isolates had identical pattern.

RESULTS

During 1998–2000 the frequency of campylobacter positive flocks on the six selected farms ranged from 16·3 to 81·5%. One hundred and eighty (89·5%) of the isolates were *C. jejuni*, 14 isolates (7·0%) were *C. coli* and 7 isolates (3·5%) belonged to other species but were not further identified (Table 1). Unfortunately, two of the *C. jejuni* isolates were lost during storage, and two were not typeable by serotyping. The remaining 176 *C. jejuni* isolates were assigned to a total of 20 different serotypes (Table 2). The most dominant serotypes were 4-complex ($n = 56$; 31·4%), 2 ($n = 32$; 18·0%), 11 ($n = 12$; 6·7%), and 12 ($n = 11$; 6·1%) (Table 2).

In three farms, 4-complex was the most frequently isolated serotype, and in one farm it was the second most frequently isolated serotype, whereas serotype 2 was the most frequently isolated in two farms; in the last farm 1,44 was the most frequently isolated serotype (Table 2).

A total of 26 different PFGE types were demonstrated. Among the 56 isolates from the local geographic area, 11 different patterns were found

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Table 2. *Serotypes of campylobacters from broilers on six farms in a small geographic area of Jutland during 1998–2000*

	Serotypes								No. of	No. of
Farm	4c	2	11	12	6,7	1,44	Others	NT*	serotypes	isolates
A	10	4		2	1		23		10	40
B	7	5	1		1		3	1	7	18
C	33	11	7	5	4		3		7	63
D	2	1	1		1	3	3	1	6	12
E	2	6	3	1	1		6		8	19
F	2	5		3	1	5	10		11	26
Total	56	32	12	11	9	8	48	2	20	178

* NT, nontypeable isolates.

Table 3. *PFGE patterns of 56* C. jejuni *serotype 4-complex isolates from broilers on six farms in a small geographic area of Jutland during 1998–2000*

	PFGE patterns											
Farm	1	2	3	4	5	6	7	8	9	10	11	Sum
A						10						10
B	1		2		1		1			1	1	7
C	1	13		1			6	11	1			33
D							2					2
E							2					2
F							2					2
Total	2	13	2	1	1	10	13	11	1	1	1	56

(designated patterns 1–11), although patterns 2, 6, 7 and 8 were clearly dominant. In four of the six farms only a single pattern was found, thus pattern 6 was only found in farm A, and at farms D–F, pattern 7 appeared to be the only pattern, and this pattern was also demonstrated on farm B and C. At farms B and C, six different patterns were found. Pattern 8 was only found at farm C (Table 3). The 13 PFGE pattern 2 isolates found on farm C were all isolated during a short period from September to December 2000, and occurred simultaneously in several broiler houses on the farm and in up to four consecutive flocks in some houses. Also the 11 PFGE pattern 8 isolates found on farm C were found during a relatively short period August 1998–January 1999 in three different houses and in up to four consecutive flocks. The 10 PFGE pattern 6 isolates found on farm A were detected during the period February 1998–December 1998 in up to four consecutive rotations in each house. These PFGE types were each found on only one farm, and obviously within a relatively short period. The 13 PFGE pattern 7 isolates were found on five different farms. However, on these farms, the isolates did not occur simultaneously. Thus, on one farm, two isolates were recovered from two different houses simultaneously in April 1998, on another farm in two different flocks in May 1998 and September 1999, respectively, on a third farm in only one flock, September 1998, and on a fourth farm in two houses simultaneously in March 1999. On the fifth farm, this pattern was isolated in two houses in consecutive rotations during spring and summer 1998.

Among the 29 strains selected at random representing the whole country, 20 different patterns were found of which pattern 2, 3, 5, 7 and 10 were also found on the six farms under study. Pattern 7 was found among seven isolates, patterns 10, 20 and 22 among two isolates each, while the remaining patterns, 2, 3, 5, 12–19, 21, 23–26, were only found in one isolate each. The diversity as calculated from their PFGE patterns among strains isolated from the whole country was considerably higher ($D_i = 0{\cdot}94$) than among the strains from the local area ($D_i = 0{\cdot}83$).

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Only four patterns namely 7, 10, 20 and 22 were found on more than one farm. Pattern 10, 20 and 22 were found at six different farms (Fig. 1). Pattern 7 was found on seven different farms distributed all over the peninsula of Jutland (Fig. 1). Most of the strains from the whole country had unique patterns, indicating a high level of diversity.

DISCUSSION

In 1998, we reported a national flock prevalence of 46·0% and a species distribution of 86% *C. jejuni* and 11% *C. coli* in Danish broilers at slaughter [7]. These figures represented the whole country and an average over a year. In the present study, we report a within flock prevalence ranging between 16·3 and 81·5%, and a species distribution in accordance with the previous report [7]. We therefore assumed that in the geographic area under study, campylobacter occurrence with respect to these parameters, was representative of the whole country. However, when looking at the serotype distribution, the six farms differed from the national picture, as the serotype 4-complex was the most frequently isolated (31·4%). This serotype was present in all six farms under investigation. Furthermore, 4-complex was the most frequently isolated serotype in three of the farms. Serotype 2 was the second most frequently isolated (18·0%), and this serotype, too, was present in all farms. In two farms serotype 2 was the most frequently isolated. In the remaining farm serotype 1,44 was most frequently isolated. Serotype 1,44 was the sixth most frequently isolated serotype (4·5%) in the study. In two Danish studies from the years 1999 and 1997 [14, 15] serotypes 2 (16·3 and 27%, respectively) and 1,44 (10·5 and 15%, respectively) were the most frequently isolated serotypes. In these studies 4-complex was the third most frequently reported serotype (9·3 and 18%, respectively).

In a recent Danish study [17], it was shown that both serotyping and PFGE were stable typing methods both yielding a high level of typeability, and that the *C. jejuni* isolates seemed to be stable during passage. Other authors have used PFGE and found that this technique yielded a reproducible and highly discriminative means of studying epidemiology and clonality of campylobacter [11, 18]. In the present study we wanted to look at clonality among campylobacter isolates, and since *C. jejuni* serotype 4-complex was the most common species and serotype, we chose to focus on those isolates. However, the conclusions are likely to be valid in a general perspective. We applied PFGE to a selection of serotype 4-complex isolates and obtained excellent results. Since all campylobacter isolates from the national surveillance programme were kept and stored at −80 °C, we had the opportunity to recover and include all known serotype 4-complex isolates for comparison. The 56 serotype 4-complex isolates from the small area were subdivided into 11 different PFGE profiles, whereas the selection of 29 serotype 4-complex isolates from the rest of the country was subdivided into 20 different patterns. In addition, certain patterns were dominant on certain farms, indicating that the infection was not introduced from various external sources but was present on the farms and persisted in their close environment and was reintroduced into the broiler houses rotation after rotation. This is in accordance with conclusions from other Danish investigations [13, 19]. However, studies performed in other countries do not entirely support this conclusion [20, 21] as their results indicate that new strains of campylobacter were often introduced into broiler houses. These studies showed that the serotype distribution within a flock sometimes changed during the production cycle, probably reflecting a constant flow of other campylobacters entering a broiler house. Whether these contradictory reports reflect differences in housing and management between countries remains to be clarified.

Most patterns were restricted to a single farm whereas others were recovered on more than one farm. The patterns that were found on only one farm usually occurred within a short period in consecutive flocks and/or simultaneously in more than one house on the farm. In contrast, PFGE pattern 7, which was found on five different farms, occurred during the period April 1998–September 1999, i.e. not simultaneously on all farms. Whether the infection was transferred between farms or the farms were infected from a common source is unknown. Since this PFGE type was also found among strains from the rest of the country, the infection may also have been introduced on each farm from independent sources.

It was not possible from our results to hypothesize on genetic stability of the strains or whether certain PFGE patterns developed into others. We used only one restriction enzyme, *Sma*I, and it is possible that a further diversity would have been demonstrated if one or more additional enzymes had been used. Thus, Perko-Mäkelä et al. [22] found that two serotype 4-complex isolates that had identical *Kpn*I types had

different *Sma*I patterns, maybe indicating genetic instability of these strains.

In a Danish study, Nielsen et al. [11] evaluated methods for subtyping of *C. jejuni*. Their study included ten 4-complex isolates from human, cattle and poultry, and these isolates were assigned to ten different PFGE profiles. These findings are similar to the findings here reported for the whole country, except for the pattern designated 7 which was found at seven different farms.

In Denmark, the chicken farmers have been paid extra money for campylobacter-free flocks since 1998. This has encouraged the establishment of hygiene barriers and other preventive measures, and it has been demonstrated that the presence of a hygiene barrier may reduce campylobacter occurrence in broiler flocks although such measures are not completely efficient [23]. However, it seems urgent to identify the reservoirs for campylobacter between rotations, the sources they are recruited from, and the vehicles that carry the bacteria into the broiler houses. Given the fragile nature of campylobacter, its susceptibility to drying, and the fact that the broilers usually do not become colonized until 2–3 weeks of age, it seems unlikely that the campylobacters survive within the house.

PFGE pattern 7 was found on five of the six farms under study, but also on other, even distant farms (Fig. 1). The reason for the apparent success of this clonal lineage and its spreading mechanisms are not clear.

In conclusion, both serotype distribution and percentage campylobacter-infected flocks varied considerably from farm to farm. We present evidence that certain campylobacter clones persisted in a confined geographical area, probably at the farm, and that the broiler houses repeatedly were infected with few *C. jejuni* clones during consecutive broiler flocks. Many clones seemed to be restricted to a single farm. New clones were also introduced and some clones appeared to be geographically more widespread; however, the sources and vehicles are yet unknown.

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