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High resolution genotyping of *Campylobacter jejuni* strains by macrorestriction analysis with *Xho*I and polymerase chain reaction targeting enterobacterial repetitive intergenic consensus sequences: can we predict the zoonotic potential of strains?

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SUMMARY

Campylobacter jejuni isolates of human, canine, feline, bovine and poultry origin were investigated for their genomic diversity using O-antigen typing ($n = 271$), *Sma*I ($n = 158$) and *Xho*I ($n = 158$) macrorestriction analysis and ERIC–PCR ($n = 107$). The O-antigens O:1/44, O:2, O:4 complex, O:37, O:40 were identified and 53·7% of the human and 56·1% of the animal strains were typable with the available antisera. Two ERIC–PCR pattern groups were generated representing human and animal strains as well as those exclusively of animal origin. *Xho*I macrorestriction analysis also distinguished 'human' and 'non-human' strain clusters, but by *Sma*I restriction mainly serotype-associated clusters were found. In conclusion, genomic differences may occur between 'human' and 'non-human' strains and this may reflect their potential to overcome the barrier from animals to humans.

INTRODUCTION

Campylobacter jejuni is recognized as an important human pathogen causing gastrointestinal disease in both developed and developing countries [1]. Reactive arthritis and polyneuropathies, such as Guillain–Barré syndrome, have been described as severe post-infectious sequelae. In the latter molecular mimicry of carbohydrate moieties of the bacterial cell surface with host cell gangliosides has been suggested as a pathogenic mechanism [2, 3]. *C. jejuni* is widespread in nature and can be isolated from gastrointestinal tracts of many mammalian and avian species as well as from contaminated surface water. Characteristically the disease arises as a sporadic infection. Contaminated and poorly cooked poultry products and raw milk are considered the major sources of human infection [4–6]. Outbreaks are rare, but sometimes occur in child day-care centres or school classes following excursions to animal farms. Pet animals carrying the pathogen may also contribute to sporadic infections due to their intimate association to man [7, 8].

A number of investigations have compared the genome characteristics of *C. jejuni* strains originating from different host species. Korolik et al. [9] detected two distinct fragment patterns of *Cla*I restricted DNA hybridizing with a specific DNA probe in strains of poultry and human origin; 71% of the strains from humans and 22% of the strains from poultry origin were characterized by the first pattern and 29% of human and 78% of poultry strains by the second pattern. Further, Duim et al. [10, 11] detected common clones present in humans and poultry with amplified fragment length polymorphism fingerprinting. This was supported by macrorestriction analyses with *C. jejuni* strains using endonucleases *Sma*I, *Sal*I, *Kpn*I and *Bam*HI which showed identical clones in humans and animals [12].

In the present investigation we have explored whether *C. jejuni* strains can be differentiated into strain types, which are found in humans as well as in

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animals and strain types exclusive to animals. This would have significant impact in the prevention of human disease. For this purpose we used different approaches of genotyping and phenotyping, (i) macrorestriction analysis with the endonucleases *Xho*I and *Sma*I, respectively, (ii) fingerprinting based on amplification of enterobacterial repetitive intergenic consensus (ERIC) DNA sequences [13], both methods targeting the entire genome and (iii) O-serotyping according to Penner & Hennessy [14]. These methods were applied to a number of *C. jejuni* strains isolated from man and different animal sources.

METHODS

Collection and cultivation of bacteria

In the course of an investigation into the presence of thermophilic campylobacters in pet animals, *C. jejuni* isolates ($n = 26$) were recovered from dogs and cats of different ages and enteric health conditions in two regions of Germany (Berlin, Northrhine-Westfalia), approximately 400 km apart, during a 12-month time period (1998–9). The specimens were collected from rectal swabs using Culturettes® (Becton Dickinson) and these were cultured on selective media within 24 h after collection. The selective media, CAT agar [15], mCCDA agar [16] and CSM agar [17] were used in order to recover also *C. upsaliensis* isolates, which were characterized in a previous study [18]. Further cultivation was performed on Mueller–Hinton agar plates containing 5% defibrinated sheep blood (MHB). Plates were incubated under microaerophilic conditions for 48–72 h at 39 °C. Stock cultures were maintained in thioglycollate broth containing 15% glycerol at −70 °C. *C. jejuni* isolates of human origin ($n = 201$) isolated from the same geographical regions during the same time period as the canine and feline isolates were kindly supplied by two diagnostic laboratories. *C. jejuni* isolates ($n = 29$) from different poultry flocks were collected independently from human, canine and feline isolates at the Institute of Poultry Diseases, Veterinary University, Hannover (6 strains; 1993–8), and the Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV), Berlin, Germany (23 isolates; 1996–9). All available information (flock, owner, slaughterhouse, time of slaughter and ribotype) indicating the relationship of the strains was taken into account. Isolates suspected to be duplicates of strains already present in the collection were omitted. Bovine strains ($n = 15$) were collected from faecal samples sent to the diagnostic laboratory of our institute from different owners.

Species determination

C. jejuni isolates were confirmed by biochemical tests according to the literature [19–22] using the following criteria: gram-negative, spiral-shaped rod morphology, requirement of microaerophilic growth conditions, cytochrome oxidase and catalase activity, hippurate hydrolysis as well as susceptibility to nalidixic acid and resistance to cephalothin. The results were confirmed using the species-specific PCR of Eyers et al. [23].

Indirect haemagglutination

Indirect haemagglutination was performed according to Penner & Hennessy [14] using rabbit antisera raised against formaldehyde-treated *C. jejuni* reference strains O:1, O:2, O:4, O:9, O:13, O:16, O:43 (Penner) and *C. jejuni* wild-type strains O:37 and O:40 as described previously [24]. Serum haemagglutination titres of 80 or less were ignored. *C. jejuni* reference strains were purchased from the Culture Collection University of Göteborg (CCUG) and the wild-type strains were serotyped at the same institute. Table 1 lists the bacterial strains used for antiserum production.

DNA preparation, DNA primers and PCR amplification

The procedures were used as described [18]. Briefly, for PCR amplification DNA was extracted by boiling bacterial suspensions for 5 min. The amplification reaction was performed using Ready-to-Go® PCR Beads (Amersham Pharmacia Biotech, Freiburg, Germany). Primer sequences were deduced from 23S rRNA genes for thermophilic *Campylobacter* species [23]. Primers, as listed in Table 2, were synthesized by Amersham Pharmacia Biotech and by MWG Biotech (Ebersberg, Germany). PCR conditions were used according to Eyers et al. [23], with slight modifications [18]. Amplified samples were analysed by electrophoresis on 1·2% agarose gels and visualized by ethidium bromide staining under UV light. ERIC–PCR [13] was performed in a volume of 25 μl in 0·5 ml tubes with an oil-overlay [18]. Each PCR included positive and negative reaction controls. Gels were

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Table 1. *Bacterial strains used for antiserum preparation*

Bacterial strain	Species	Serotype	Origin
10935	*C. jejuni*	O:1	Culture Collection University of Göteborg (CCUG); reference strain
10936	*C. jejuni*	O:2	CCUG; reference strain
10938	*C. jejuni*	O:4	CCUG; reference strain
10945	*C. jejuni*	O:13	CCUG; reference strain
10947	*C. jejuni*	O:16	CCUG; reference strain
12783	*C. jejuni*	O:43	CCUG; reference strain
L2H	*C. jejuni*	O:37	Germany; wild type strain; origin unknown
1834	*C. jejuni*	O:40	Germany; wild type strain; human origin

Table 2. *Primers and target genes used for PCR*

Target gene	Primer	Primer sequence	Reference
Thermophilic camplobacter	Therm 1 Therm 2	5′-TATTCCAATACCAACATTAGT-3′ 5′-CGGTACGGGCAACATTAG-3′	Eyers et al. [23] Erratum [8]
C. jejuni (23S rRNA)	Jej 2 Jej 1 Therm 3	5′-GTAAATCCTAATACAAAGCT-3′ 5′-TAAATCCTAGTACGAAGCT-3′ 5′-TAAAGTAAGTACCGAAGCTG-3′	Erratum [8] Eyers et al. [23]
Enterobacterial intergenic repetitive consensus sequence	ERIC I ERIC II	5′-ATGTAAGCTCCTGGGGATTCA-3′ 5′-AAGTAAGTGACTGGGGTGAGCG-3′	Versalovic et al. [36]

photographed with a digital camera system (Herolab, Wiesloch, Germany).

Macrorestriction analysis using pulsed-field gel electrophoresis (PFGE)

PFGE was performed using a CHEF DR III apparatus (Biorad, Munich, Germany) [18]. The pulse intervals for *Sma*I were ramped from 5–10 s linearly for 4 h, 10–40 s for 14 h and 50–60 s for 4 h; the pulse interval for *Xho*I was 0·3–12 s linearly for 24 h. Reference DNA of the *C. jejuni* strain CCUG 10938 digested with *Sma*I and *Xho*I, respectively, was run on each gel. Molecular weight standard λ concatamers (MW = 48·5 kb; Roche Diagnostics, Mannheim, Germany) were run on three lanes (both edges and middle) of each gel. Gels were stained with ethidium bromide, viewed under UV light and photographed on polaroid films.

Computational analysis

The electrophoretic patterns of ERIC–PCR experiments were analysed using Gelcompar 4.1 Software® (Applied Maths BVBA, Kortrijk, Belgium). Genetic similarities between isolates based on band positions and brightness of bands were calculated using the algorithm of Ward [25] and the Pearson product–moment correlation coefficient. The reproducibility of profiles was $\geqslant$ 94%.

Photographs of PFGE experiments were scanned and similarities were calculated using the same software. Variations in the intensity of bands were ignored. The algorithm of Ward [25] and the Dice coefficient [26] with a maximum tolerance of 1·0% and optimization of 0·5% for both enzymes were used. With these parameters the reproducibility of band profiles generated from eight duplicate strains restricted with *Sma*I was $\geqslant$ 95·5% and from six duplicate strains restricted with *Xho*I was $\geqslant$ 95·8%. The statistical method applied to determine the significance of the defined similarity groups was the 'Jackknife' method (GelCompar II version 2.50, comparative analysis of electrophoresis patterns, version 2.50 (Applied Maths).

RESULTS

Distribution of O-antigens

Of 201 *C. jejuni* isolates of human origin serotyped according to their O antigens (Table 3), 44·7% belonged to the serogoups O:1/44, O:2 and the complex serogroup O:4 (13/16/43), 4% to O:37 and 5% to O:40; 46·3% were not typable with the available

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Table 3. *O-antigen specificities of* C. jejuni *isolates from canine, feline, bovine, poultry and human origin*

Host	Number of isolates	% of isolates belonging to serotype					
		O:1/44	O:2	O:4 complex	O:37	O:40	O n.t.
Man	201	14·4	17·9	12·4	4·0	5·0	46·3
Cattle	15	20	26·7	20	0	0	33·3
Dog/cat	26 (22/4)	7·7	7·7	30·8	0	3·8	50·0
Poultry	29	10·3	27·6	6·9	6·9	0	48·3

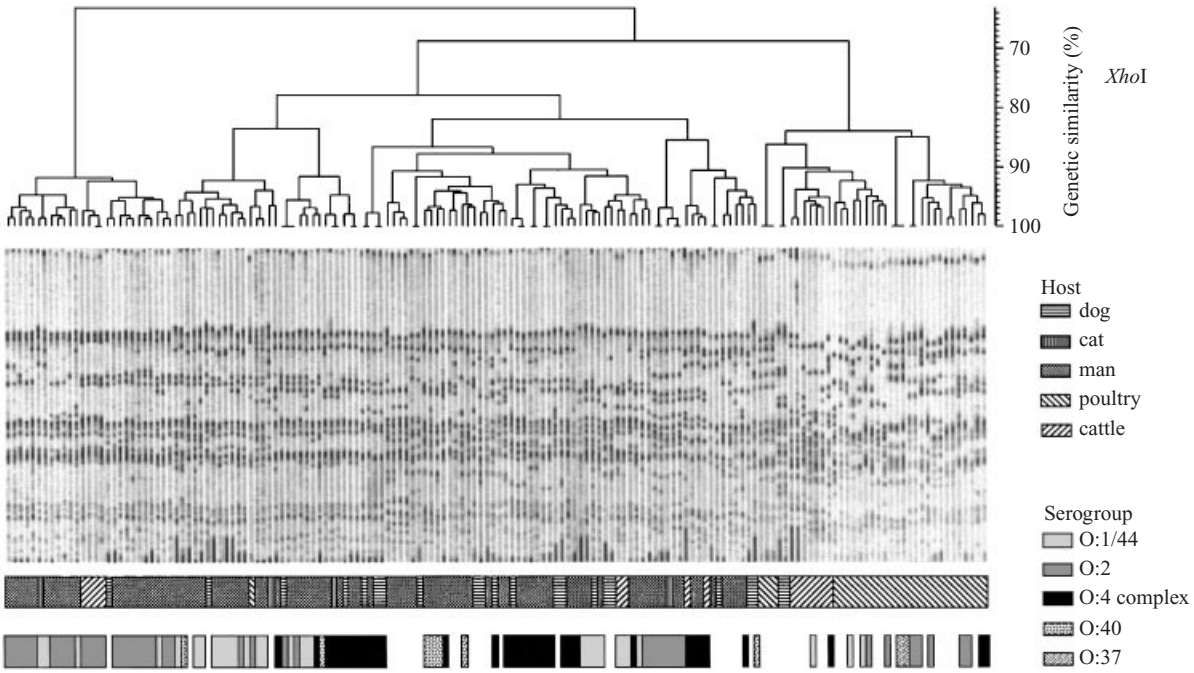

Fig. 1. Genetic similarities of 158 *C. jejuni* strains isolated from human, canine, feline, bovine and poultry origin based on macrorestriction analysis using endonuclease *Xho*I.

antisera. Of 22 canine and 4 feline isolates, 46·2% fell in the serotypes O:1, O:2 and O:4 complex, 3·8% in O:40 and 50·0% were not typable. For 29 poultry strains, 44·8% were identified as O:1/44, O:2, O:4 complex, 6·9% each as O:37 and O:40, and 48·3% were not typable. No strain of serotype O:37 was found. The majority of 15 bovine isolates (66·7%) were of serogroups O:1/O:44, O:2 and O:4 complex but the remainder were not typable (Table 3).

Macrorestriction analysis

Eighty-eight human isolates were selected for DNA macrorestriction analysis ensuring that the most prevalent serotypes (O:1/44, O:2, O:4 complex) and the untypable strains were represented in comparable numbers. Isolates from the other sources also examined included 22 canine and 4 feline, 29 poultry and 15 bovine isolates. Two major PFGE groups were differentiated by *Xho*I restriction (Fig. 1). The first group contained all 88 human, 4 feline, 20 of 22 canine and 7 of 15 bovine isolates and only 1 of 29 poultry isolates. The second group comprised all the other poultry isolates, 8 of 15 bovine and 2 of 22 canine isolates. There was no association between PFGE group with O-antigen reactions of the strains.

*Sma*I macrorestriction analysis (Fig. 2) revealed two main clusters. The first contained 79 isolates which were subdivided into serotype-associated subclusters, one O:4 complex-associated and two O:1/44/O:2 associated. The isolates within the latter two subclusters were randomly distributed. In the second main cluster a rather heterogeneous group of 79 isolates was assembled, 59·0% of which were not typable with the

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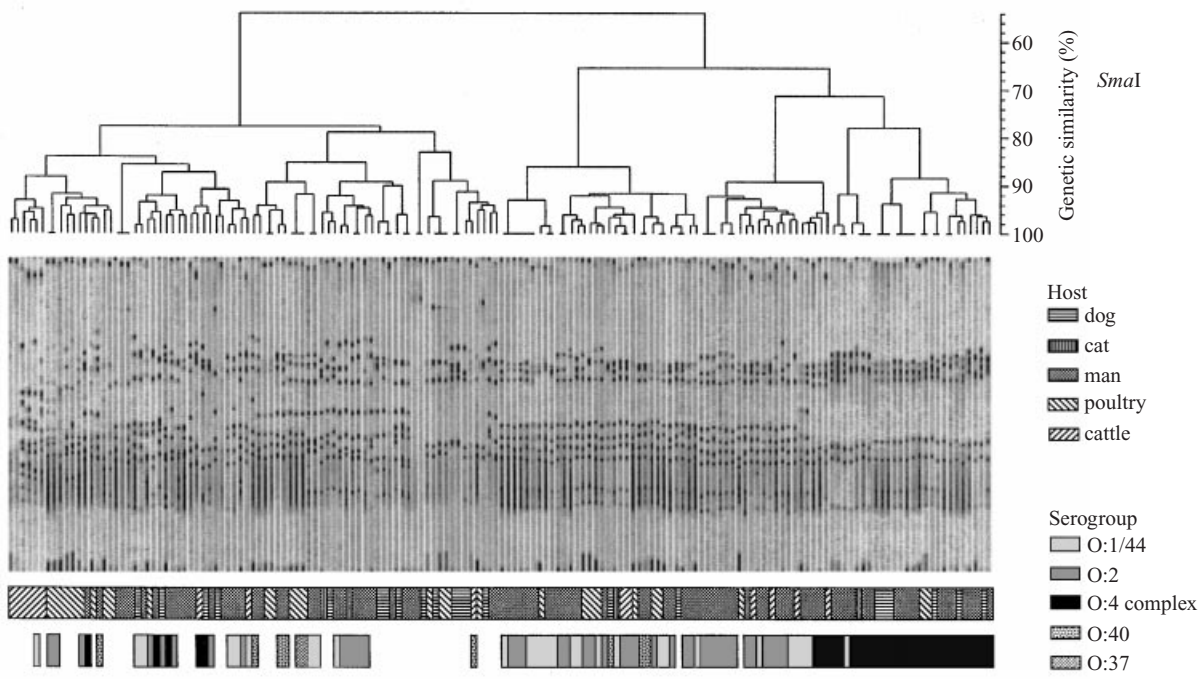

Fig. 2. Genetic similarities of 158 *C. jejuni* strains isolated from human, canine, feline, bovine and poultry origin based on macrorestriction analyses using endonuclease *Sma*I.

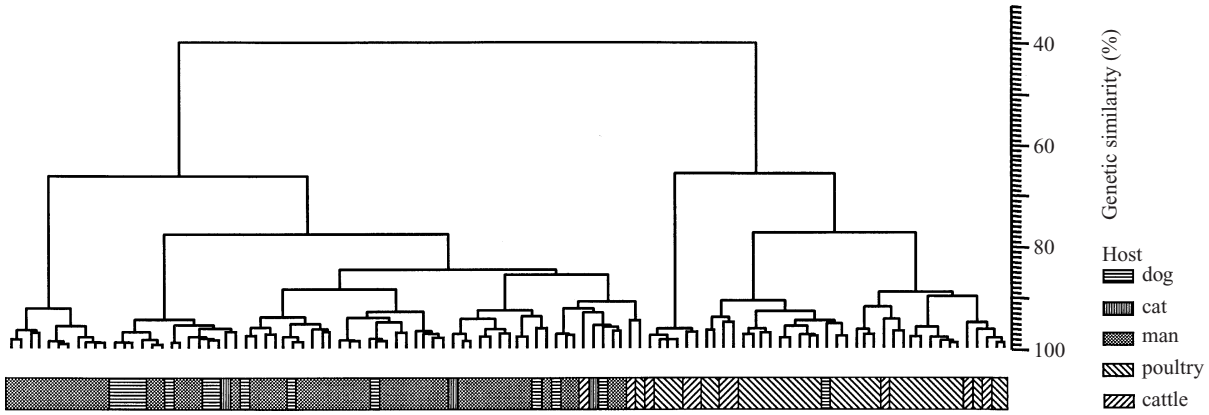

Fig. 3. Genetic similarities of 107 *C. jejuni* strains isolated from human, canine, feline, bovine and poultry origin based on ERIC–PCR patterns.

available antisera. In contrast, the aforementioned first cluster contained only 2·5 % of untypable isolates. The geographic origin of the strains was not reflected by the dendrograms.

ERIC fingerprint analysis

Forty-eight randomly chosen *C. jejuni* isolates of human, 18 canine and feline, 14 bovine and 27 poultry isolates characterized by macrorestriction analysis were subjected to ERIC fingerprinting. Two main clusters were generated exhibiting only 40 % similarity (Fig. 3). Cluster I consisted of 26 of 27 poultry isolates, 12 of 14 bovine as well as 1 of 18 canine and feline isolates, while cluster II contained the total number of 48 human, 17 of 18 canine and feline isolates as well as 1 of 27 poultry and 2 of 14 bovine isolates. The geographic origin of the strains was not reflected by the dendrogram.

Statistical significance

Table 4 shows results the statistical calculations assessing the stability of the defined groups (significance). The percentages of identifications for members of a group are given in the respective columns. In

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Table 4. *Internal stability* (*significance*) *of the defined strain clusters in association with host origin calculated using the Dice* (*A*, *C*, *E*) *and the Pearson coefficient* (*B*, *D*, *F*), *respectively*. *The percentages of correct identifications are displayed in the matrices*

	Man	Poultry	Cattle	Dog	Cat	Man	Poultry	Cattle	Dog	Cat
(A) *Sma*I						(B)				
Man	80·7	31·1	33·3	50·0	75·0	81·8	27·6	26·7	40·9	50·0
Poultry	6·8	48·3	0·0	13·6	0·0	6·8	69·0	6·7	9·1	0·0
Cattle	4·5	6·9	60·0	4·5	25·0	3·4	0·0	66·7	4·5	0·0
Dog	5·7	13·8	6·7	31·8	0·0	5·7	0·0	0·0	40·9	50·0
Cat	2·3	0·0	0·0	0·0	0·0	2·3	3·4	0·0	4·5	0·0
(C) *Xho*I						(D)				
Man	85·2	10·3	13·3	59·1	100·0	84·1	3·4	0·0	54·5	75·0
Poultry	0·0	89·7	0·0	4·5	0·0	1·1	93·1	6·7	4·5	0·0
Cattle	0·0	0·0	86·7	4·5	0·0	1·1	0·0	86·7	0·0	0·0
Dog	11·4	0·0	0·0	31·8	0·0	9·1	3·4	6·7	40·9	0·0
Cat	3·4	0·0	0·0	0·0	0·0	4·5	0·0	0·0	0·0	25·0
(E) ERIC						(F)				
Man	79·2	16·7	27·3	66·7	66·7	75·0	0·0	9·1	60·0	66·7
Poultry	4·2	70·0	27·3	6·7	0·0	0·0	96·7	54·5	0·0	0·0
Cattle	0·0	6·7	45·5	0·0	0·0	0·0	3·3	27·3	0·0	0·0
Dog	14·6	6·7	0·0	26·7	33·3	25·0	0·0	9·1	40·0	33·3
Cat	2·1	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0

addition to the dendrogram calculations here the groupings were also based on the Pearson coefficient for *Sma*I and *Xho*I (Table 4A, C, E) and the Dice coefficient for ERIC–PCR (Table 4B, D, F).

DISCUSSION

Infectious agents seeking to manage the transition from one host to another need to be able to adapt to the new host. Genotypic differences of isolates from different hosts or reservoirs may indicate a decreased likelihood of successful transmission of infection between them. These differences may help to reveal the probability of sources for human and animal infections for diagnostic purposes. Human infections with *C. jejuni* are generally thought to originate from the consumption of contaminated undercooked poultry products or raw milk [27]. Indeed, in several epidemiological studies profound differences between *C. jejuni* strains present in man and animals have not been detected [5, 12, 28–30]. These investigations were performed using multilocus enzyme electrophoresis, serotyping, amplified-fragment length polymorphism genotyping, PCR–RFLP analyses of the flagellin gene or macrorestriction analyses with the endonucleases *Sma*I, *Sal*I, *Kpn*I and *Bam*HI. In contrast, data obtained by Duim et al. [10] using amplified fragment length polymorphism analysis, indicated that certain *C. jejuni* genotypes may be more frequently associated with human disease than others and that a number of genotypes present in human isolates were not detected in chicken strains.

In the present study four typing methods exhibiting different discriminatory power were applied to differentiate *C. jejuni* strains to the subspecies level, serotyping, ERIC–PCR and macrorestriction analysis with *Xho*I and *Sma*I. The repetitive ERIC sequences provide useful targets for epidemiological analysis, since they are present in multiple copies at different positions in the genome [13]. They therefore permit simultaneous scanning for DNA polymorphisms at multiple genome loci. There are 24 *Xho*I recognition sites scattered over the genome of *C. jejuni* strain NCTC 11168 [31]. This endonuclease was thus expected to yield complex and discriminatory restriction profiles. *Sma*I normally generates less than 10 fragments of the *C. jejuni* chromosome and its discriminatory potential is therefore limited for bacterial strain typing by pulsed-field gel electrophoresis [32]. Nevertheless, *Sma*I has been used frequently to detect clonal groups of *C. jejuni* at the subspecies level in association with special serotypes. Indeed, the serotypes O:1, O:4 and O:55 have been shown to be clonal by this method [33–35]. For *C. sputorum* a clonal population structure was also found with respect to *Sma*I restriction profiles [35].

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Two of the genotypic approaches, macrorestriction with *Xho*I and ERIC–PCR, accentuated genotypic relatedness of strains in association with their host origin better than the third genotypic method, macrorestriction with *Sma*I. By macrorestriction with *Xho*I three main clusters were generated. Two of these were relatively closely related and contained the human strains in addition to the vast majority (88·8%) of canine and feline strains, some of the bovine and less than 10% of the poultry strains. The third cluster comprised the vast majority (92·3%) of poultry and part of the bovine strains. ERIC–PCR amplification was the most discriminatory method to differentiate between strains of the clusters 'human' and 'non-human' in this study, as these clusters shared only 40% similarity.

In contrast, groups associated with O-antigens, at least O:1, O:2 and O:4-complex, were generated to some extent by macrorestriction with *Sma*I. Most of the strains belonging to the O antigens O:1/44 and O:2 fell in two very closely related clusters, each cluster containing both O:1/44 and O:2 strains randomly mixed, which is in agreement with previously published results [34, 36]. The close relationship of O:1 and O:2 positive strain clusters may perhaps reflect the close structural similarity of the two O-antigens [37]. Another cluster contained most of the strains positive for O:4 complex, and one strain cluster comprised the vast majority of 'untypable' strains, which did not belong to the serotypes O:1/44, O:2, O:4 complex, O:37 and O:40. The high degree of heterogeneity of this 'untypable' strain cluster may indicate that strains with a large number of different O-antigens are assembled in this group. Analysis of the specific recognition sequence of *Sma*I in *C. jejuni* NCTC 11168 [31] show that 9 of 15 recognition sites are localized within the three copies of the highly conserved rRNA genes, but outside the LOS-gene region. Thus, some processes of gene arrangement in the history of taxonomic evolution and O-antigenic diversification of the genus *Campylobacter* into serotypes may have emerged in parallel leading to this phenomenon.

The significance of the groups displayed in the dendrograms is emphasized by statistical calculations, by which the internal stability (significance) of the groups was determined. The principle of the Jackknife method is to take away one entry (one at a time) from the analysis, consider it as 'unknown' and to identify this entry against the different groups, calculating average similarities or maximal similarities with each group. In this investigation maximal similarities were calculated for all entries. The percentage of cases that are identified to the group they were assigned, is a measure of the internal stability (significance) of that group. The results suggest that strains of human origin form a rather stable group with at least 75% correct assignment of strains, which comprises at least 40·9% of canine and feline isolates as 'incorrectly' assigned strains. Therefore, a considerable part of the canine, and even more feline isolates, seem to belong to the same genotypic pool as human isolates and may therefore cause infection in man. Furthermore, these results suggest that the majority of poultry strains belong to a genotypic pool separate from the human. This does not necessarily indicate that poultry strains do not cause infection in man, but it suggests that only a certain (perhaps small) subset of poultry strains is able to infect man ('zoo-anthroponotic potency') while the others stay confined to the animal niche. In this investigation, fortunately or unfortunately, the vast majority of poultry isolates fell in the non-zooanthroponotic subset. Finally, bovine like poultry strains but to a lesser extent, form subsets separate from human strains. The analysis of the *Sma*I-generated clusters (Table 4A, B) confirmed that, as expected from the dendrogram, host association was the weakest by this approach. The result that the geographic origin of the strains did not have any influence on their relationship reflected by their positions in the dendrograms was surprising. However, no indication was found that strains from one of the areas were more closely related to each other than the strains from different areas.

In conclusion, this study shows that, by using different typing methods, different aspects of the interrelationship of bacterial strains within a species may be emphasized as host adaptation may have emerged across taxonomic lines. Furthermore, including one or the other parameter (Table 4; band position and intensity *vs*. band position only) into the analysis leads to slightly different results. Inclusion of the intensity of bands as an additional parameter in the analysis gives a clearer separation of the host-associated groups than the band position alone. The general implication of these findings for host adaptation cannot be answered at this moment as this investigation was performed with a number of strains, the majority of which were collected during a limited time period and from relatively circumscribed geographic areas. It may therefore reflect only a transient moment in the evolution of bacterial–mammalian interrelationship. More detailed knowledge of the genome characteristics of *C. jejuni* strains from different hosts will be necessary to esti-

mate the potential of strains to cause disease and the use of genotyping methods for predictive infectious disease studies.

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