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Genetic diversity in *Helicobacter pullorum* from human and poultry sources identified by an amplified fragment length polymorphism technique and pulsed-field gel electrophoresis

J.R. Gibson[1], M.A. Ferrus[2], D. Woodward[3], J. Xerry[1] and R.J. Owen[1]

[1]Laboratory of Enteric Pathogens, Central Public Health Laboratory, London, UK; [2]Departamento de Biotecnologia, Universidad Politecnica, Valencia, Spain; [3]Laboratory Centre for Disease Control, Bureau of Microbiology, Federal Laboratories for Health Canada, Winnipeg, Canada

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J.R. GIBSON, M.A. FERRUS, D. WOODWARD, J. XERRY AND R.J. OWEN. 1999. *Helicobacter pullorum* was first isolated from the faeces and carcasses of poultry and has been associated with human gastroenteritis. The aim of this study was to examine interstrain genetic diversity within *H. pullorum*. Two fingerprinting techniques were used: amplified fragment length polymorphism (AFLP) and pulsed field gel electrophoretic (PFGE) analysis. The 20 strains examined were from four countries and comprised 13 human isolates and seven poultry isolates. Their identity was confirmed by a species-specific PCR assay. The human and poultry isolates had distinct genotypes and most strains showed a high degree of genetic diversity. Genotyping also indicated a clonal origin for two strains from the same poultry flock, and established a close relatedness between three chicken carcass isolates from a processing plant. It is concluded that these two genotyping techniques will provide a useful basis for future epidemiological investigations of *H. pullorum* in poultry, and may provide a link with its possible causal role in human gastrointestinal infections.

INTRODUCTION

The genus *Helicobacter* was created in 1989 (Goodwin *et al*. 1989) and has since expanded to include about 20 species of microaerobic Gram-negative bacteria that are morphologically diverse and have been isolated from gastric tissue or intestinal contents of a wide range of animal and bird species, as well as man (Owen 1998).

The main defining feature of the genus *Helicobacter* is the presence of a sheathed flagellum. In general, species associated with gastric mucosa, such as *H. pylori*, are urease-positive, and those species associated with intestinal mucosa, such as *H. cinaedi*, tend to be urease-negative. *Helicobacter pullorum*, a urease-negative organism, was described by Stanley *et al*. (1994) and although its flagellum lacked a sheath, it was classified as *Helicobacter* on the basis of 16S rRNA phylogenetic analysis. Subsequently, the organism was found to be widely distributed on the carcasses and in the caecal contents of asymptomatic poultry at slaughter (Burnens *et al*. 1996; Atabay *et al*. 1998), and was isolated from the livers and intestinal contents of laying hens with lesions suggestive of vibrionic hepatitis (Burnens *et al*. 1996; Stanley *et al*. 1994). *Helicobacter pullorum* was also isolated from faeces of humans with gastroenteritis (Burnens *et al*. 1994; Stanley *et al*. 1994; Steinbrueckner *et al*. 1997), and from bile in human cases of primary sclerosing cholangitis (Fox *et al*. 1998), although it was unclear if the organism had a causal role in these infections.

In view of the increasing number of reports on the isolation of *H. pullorum* from human and chicken specimens, the aim of the present study was to develop genomic-based methods for discriminating strains within *H. pullorum*, and then to apply the methods to compare isolates from human infections with those isolated from poultry. Such information would be an important step in establishing if poultry might be a

Correspondence to: Dr Robert Owen, Helicobacter Reference Unit, Laboratory of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, UK (e-mail: rowen@phls.nhs.uk).

significant source of the human infections, and in the recognition of a potential new food-borne agent of enteric disease. To date, reports on *H. pullorum* have been concerned with the primary isolation of strains and identification to species level, but not with the subtypic characterization of individual isolates of *H. pullorum*. Whole cell protein profiles provided some indication of phenotypic diversity within the species although overall, they are conserved and have been used as a means of species identification rather than for distinguishing between isolates (Stanley *et al*. 1994; Atabay *et al*. 1998).

In the present study, strains of *H. pullorum*, isolated from both human and poultry sources in widely different geographical locations, were investigated using two different molecular techniques that sample variation throughout the genome. The amplified fragment length polymorphism (AFLP) technique developed recently for genotyping within *H. pylori* (Gibson *et al*. 1998), and pulsed field gel electrophoretic (PFGE) profiling developed for campylobacters (Gibson *et al*. 1994, 1995), were used to determine the level of intraspecific genomic diversity.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The 20 isolates of *H. pullorum* examined in this study included 13 human and seven poultry isolates from four countries isolated between 1991 and 1997. Details of the original isolation and identification of the strains have been reported elsewhere (Table 1). Cultures of *H. pullorum* were preserved in lyophilized form or in 10% (v/v) glycerol in Nutrient Broth (Oxoid) over liquid nitrogen or at −80 °C. All strains were cultured on blood agar (Columbia agar base (Oxoid), with 10% (v/v) defibrinated horse blood), at 37 °C, under microaerobic conditions (4% O_2, 5% CO_2, 3% H_2 and 88% N_2) in a Variable Atmosphere Incubator (Don Whitley Scientific Ltd, Shipley, UK).

DNA extraction and species confirmation using PCR

DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method according to the DNA miniprep protocol of Wilson (1987). The precipitated DNA was dissolved in 50–100 μl distilled water and the concentration and purity of the samples were determined by absorbance readings at 230, 260 and 280 nm. Species identity was confirmed using the *H. pullorum* species-specific 16S rRNA gene PCR assay (Stanley *et al*. 1994). In brief, the primer sequences were: 5′ ATG AAT GCT AGT TGT TGT CAG 3′ (forward) and 5′ GAT TGG CTC CAC TTC ACA 3′ (reverse), and the cycling conditions involved an initial denaturation of 95 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min 15 s. The 448 bp product was detected by ethidium bromide staining as described previously (Stanley *et al*. 1994).

Restriction endonuclease digestion and ligation of adaptors for AFLP

An aliquot containing 4 μg DNA was digested overnight (16 h) at 37 °C with 24 U of *Hin*d III (NBL Gene Sciences, Northumberland, UK) in the buffer provided (50 mmol l^{-1} Tris-HCl, pH 8·3, 50 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} dithiothreitol) with 5 mmol l^{-1} spermidine trihydrochloride (Sigma) added in a final volume of 20 μl. A 5 μl aliquot containing 1 μg digested DNA was used in a ligation reaction containing 0·2 μg of each adaptor-oligonucleotide (detailed below), 1 U T4-DNA ligase (Boehringer Mannheim) and single-strength ligase buffer (66 mmol l^{-1} Tris-HCl, pH 7·5, 5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} dithiothreitol, 1 mmol l^{-1} ATP) in a final volume of 20 μl held at 37 °C for 3–4 h. The complementary oligonucleotide sequences used for the adaptor, 5′ACGGTATGCGACAG 3′ (ADH1) and 5′ AGC TCTGTCGCATACCGTGAG 3′ (ADH2) (Gibson *et al*. 1998), were synthesized by PE-Applied Biosystems, Warrington, UK. These oligonucleotides incorporated an additional base pair in the restriction site in order to eliminate it after ligation of the adaptor to the restricted fragment, as previously described (Gibson *et al*. 1998).

PCR template preparation for AFLP

The ligated DNA sample was heated to 80 °C for 10 min to inactivate the T4 ligase, then diluted (1/5 or 1/10) in distilled water. A 5 μl aliquot of diluted DNA was used as template in a 50 μl PCR mix.

PCR primers and PCR for AFLP

The four primers used in the PCR were 5′GGTATGCGACAGAGCTTX 3′ where the final 3′ base (X) was A for primer H1-A; T for primer H1-T; C for primer H1-C; G for primer H1-G. They were synthesized by MWG-Biotech UK Ltd (Milton Keynes, UK). Amplification reactions were performed using an Omnigene thermocycler (Hybaid Ltd, Ashford, UK), in a total volume of 50 μl, overlaid with 60 μl mineral oil, containing 5 μl template DNA (described above), 2·5 mmol l^{-1} $MgCl_2$, 300 ng of a single primer and 0·2 μl (1 U) *Taq* DNA polymerase (Gibco) in 1 × PCR buffer provided. The amplification cycles were: an initial denaturing step of 94 °C for 4 min, followed by 33 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2·5 min. Amplified fragments (6·5 μl aliquots) were separated by electrophoresis in a 1·5% (w/v) agarose gel (Ultrapure Agarose, Gibco BRL) in TBE buffer (90 mmol l^{-1} Tris, 90 mmol l^{-1} boric acid,

Table 1 *Helicobacter pullorum* strains studied

Strain*	Other strain nos	Source	Geographic origin	Reference	Lane number in Fig. 1	Lane number in Fig. 3
Chicken isolates						
NCTC 12824†	H152-C5	Healthy broiler chicken	Switzerland	Stanley *et al.* 1994	1	1
NCTC 12825	H154-C5	Healthy broiler chicken	Switzerland	Stanley *et al.* 1994	2	2
H342	HCF 14	Factory eviscerated chicken.	UK	Atabay *et al.* 1998	16	12
H343	HCF 7	Factory eviscerated chicken.	UK	Atabay *et al.* 1998	17	13
H344	DCC 5B2	Factory eviscerated chicken.	UK	Atabay *et al.* 1998	18	14
H495	CCUG 33840; H59–94	Laying hen, vibrionic hepatitis	Switzerland	Burnens *et al.* 1996	19	17
H496	CCUG 33842	Laying hen, vibrionic hepatitis	Switzerland	Burnens *et al.* 1996	20	18
Human isolates						
NCTC 12826	CCUG 33838	Gastroenteritis and hepatitis	Switzerland	Burnens *et al.* 1994	3	15
NCTC 12827		HIV-positive patient with gastroenteritis	Switzerland	Burnens *et al.* 1994	4	3
H418	case 1	Immunodeficient patient with diarrhoea	Germany	Steinbrueckner *et al.* 1997	6	4
H419	case 2	Gastroenteritis	Germany	Steinbrueckner *et al.* 1997	7	5
H436	LCDC 15115	Gastroenteritis	Canada	Melito *et al.* 1998	8	6
H437	LCDC 15136	Gastroenteritis	Canada	Melito *et al.* 1998	9	7
H438	LCDC 16143	Gastroenteritis	Canada	Melito *et al.* 1998	10	19
H439	LCDC 16767	Gastroenteritis	Canada	Melito *et al.* 1998	11	20
H440	LCDC 16936	Gastroenteritis	Canada	Melito *et al.* 1998	12	8
H441	LCDC 17264	Gastroenteritis	Canada	Melito *et al.* 1998	13	9
H442	LCDC 17353	Gastroenteritis	Canada	Melito *et al.* 1998	14	10
H443	LCDC 17388	Gastroenteritis	Canada	Melito *et al.* 1998	15	11
H494	CCUG 33839	Gastroenteritis, concomitant *Campylobacter jejuni* infection	Switzerland	Burnens *et al.* 1994	5	16

* Strains isolated from faeces or caecal contents with the exceptions of H344 (from carcass washings) and H496 (from bile).
† Type strain

2 mmol l^{-1} EDTA), and were stained with ethidium bromide (0·5 μg ml^{-1}).

Pulsed-field gel electrophoresis

The preparation and subsequent enzyme digestion of bacterial DNA for PFGE were as described previously (Gibson *et al*. 1994). *Sac*II or *Sma*I restriction fragments were electrophoretically separated in agarose gels (1% w/v) at 200 V, 14 °C for 23 h with ramped pulse times from 2 s to 12 s. Four strains (H438, H439, H494 and H496), which initially produced faint PFGE banding patterns, were pre-treated with formalin, as previously described (Gibson *et al*. 1994), in order to obtain clear profiles.

Numerical analysis of the electrophoretic patterns

The AFLP fragments of each strain were sized using Microsoft Excel 5·0 according to the method described by Lorenz *et al*. (1997). Thirty-nine differently sized bands were identified in the profiles of the 20 strains using primer HI-C and 41 bands in those generated by primer HI-G. Each strain profile was screened for every band possible for a particular primer, and positive (presence) or negative (absence) results were recorded. Numerical analysis of the patterns was performed using the NTSYS-PC program (Applied Biostatistics Inc., Setauket, NY, USA). Similarities between strains were estimated using the Dice coefficient (negative matches excluded) and strains were clustered by the unweighted pair group method using arithmetic averages (UPGMA).

RESULTS

Identification in species-specific PCR assay

The *H. pullorum* reference (type) strain, NCTC 12824, and the 19 other strains all gave the expected 448 bp amplicon in the *H. pullorum* species-specific assay.

Primer selection for AFLP

Preliminary tests for all four primers were performed on a subset of six strains of *H. pullorum*. Primers HI-C and HI-G, with terminal 3′ bases of C and G, respectively, both produced fragment profiles that were suitable for visual analysis (Fig. 1a and 1b). Use of primer HI-A produced similar patterns for all six strains, and primer HI-T led to the production of many poorly resolved fragments (results not shown).

AFLP profiling and numerical analysis

All 20 strains were fingerprinted by AFLP using *Hin*dIII to digest the DNA. Primers HI-C and HI-G produced profiles with between 13 and 21 bands, sized from approximately 180–2000 bp (Fig. 1). Isolates from different geographical sources or different animal hosts each gave unique patterns. However, strains NCTC 12824 and NCTC 12825, isolated from the faeces of two healthy broiler chickens from the same flock in Switzerland, showed identical profiles with all four primers (lanes 1 and 2, Fig. 1a and 1b show results for primers HI-C and HI-G). Likewise, three strains isolated from poultry carcasses directly after evisceration at a processing plant in the UK (strains H432, H433 and H434, Table 1) showed identity with all four primers (lanes 16–18, Fig. 1a and 1b show results for primers HI-C and HI-G).

Primer HI-C profiles contained between 13 and 17 bands (Fig. 1a), two of which (one at 1087 bp and the other at 306 bp) were common to all strains. Numerical analysis of the profiles derived from the use of primer HI-C showed that most strains (18/20) grouped at the 70% similarity level (Fig. 2a). However, two strains (H438 and H439) showed only 33% similarity with the remaining 18 strains. Both these strains were from Canadian patients and they grouped in a single cluster at the 73% similarity level.

Primer HI-G profiles contained between 14 and 21 bands and all strains shared a band of 514 bp (Fig. 1b). Most strains showed 70% similarity in numerical analysis of the AFLP banding patterns (Fig. 2b), although one strain (H496) was 60% similar to the main group and the two strains from the Canadian patients (H438 and H439), which were separated from the others in the HI-C primer derived patterns, were only 41% similar to the others. These two isolates grouped in a single cluster at the 82% level so were more similar to each other than to any other strain in the set.

PFGE profiling

DNA fingerprints were obtained from all 20 isolates of *H. pullorum* when PFGE was performed after *Sac*II digestion (Fig. 3a), and from 18 of the 20 isolates following *Sma*I restriction (Fig. 3b). The two isolates (H438 and H439) with DNA that did not cut with *Sma*I were the same two which showed 41% or less similarity to other strains in the AFLP analysis. Strains NCTC 12824 and NCTC 12825, which were identical on AFLP analysis, also had identical *Sac*II profiles, and differed in the position of just one band on *Sma*I analysis. A band of approximately 147 kbp in the NCTC 12824 profile was lost and a smaller band was gained at approximately 145 kbp in strain NCTC 12825 (Fig. 3b, lanes 1 and 2). The restriction patterns of the three poultry isolates from the UK that showed identity on AFLP analysis were identical to each other on PFGE (*Sma*I and *Sac*II) analysis. Little similarity

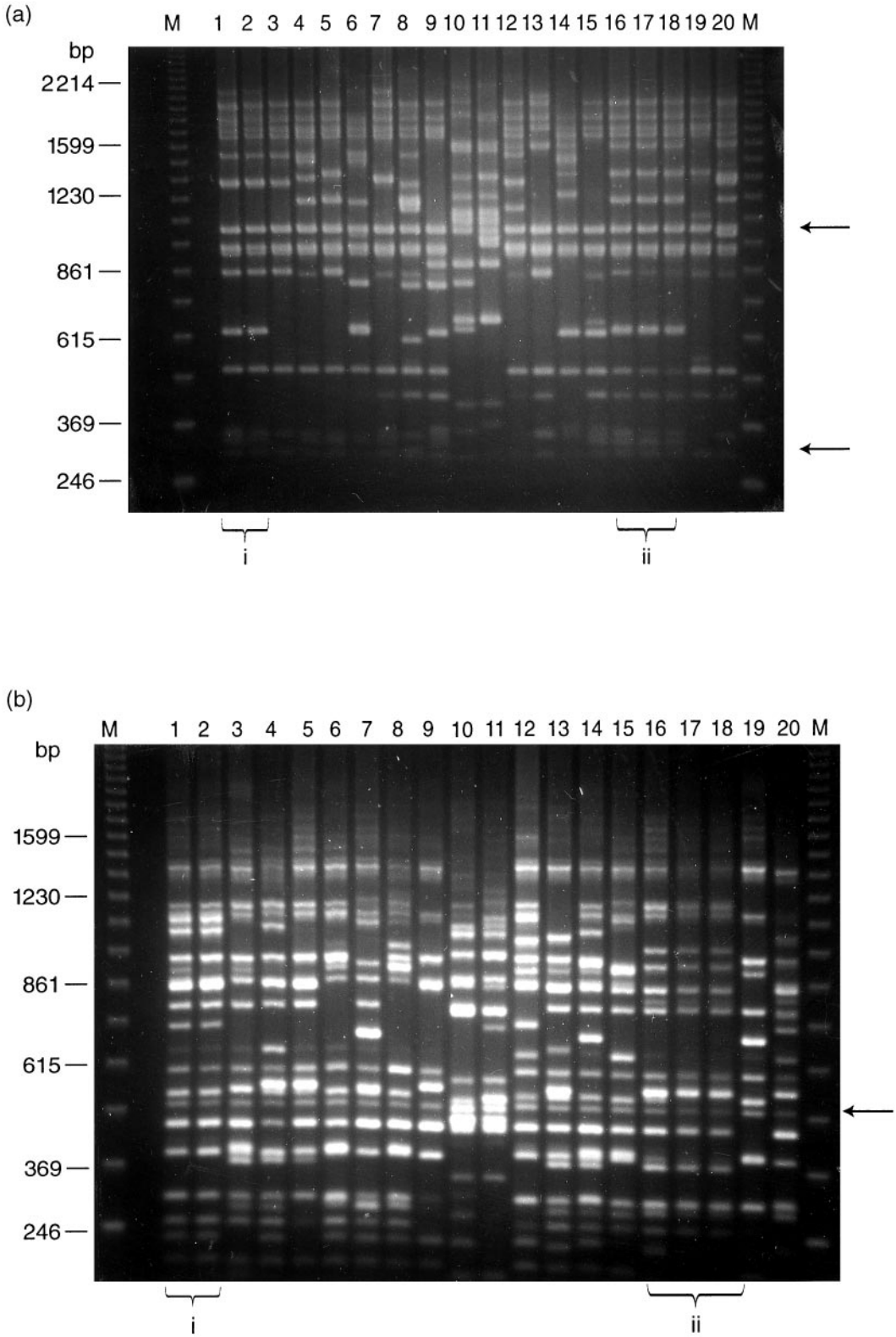

Fig. 1 AFLP banding profiles of strains of *Helicobacter pullorum* produced using (a) primer HI-C and (b) primer HI-G. Lane contents are listed in Table 1. Strains showing identity are bracketed: (i) NCTC 12824 and NCTC 12825; (ii) H342, H343 and H344. DNA size standards (123 bp marker; Gibco BRL) are in lanes marked M. Arrows mark the positions of bands common to all strains (see text)

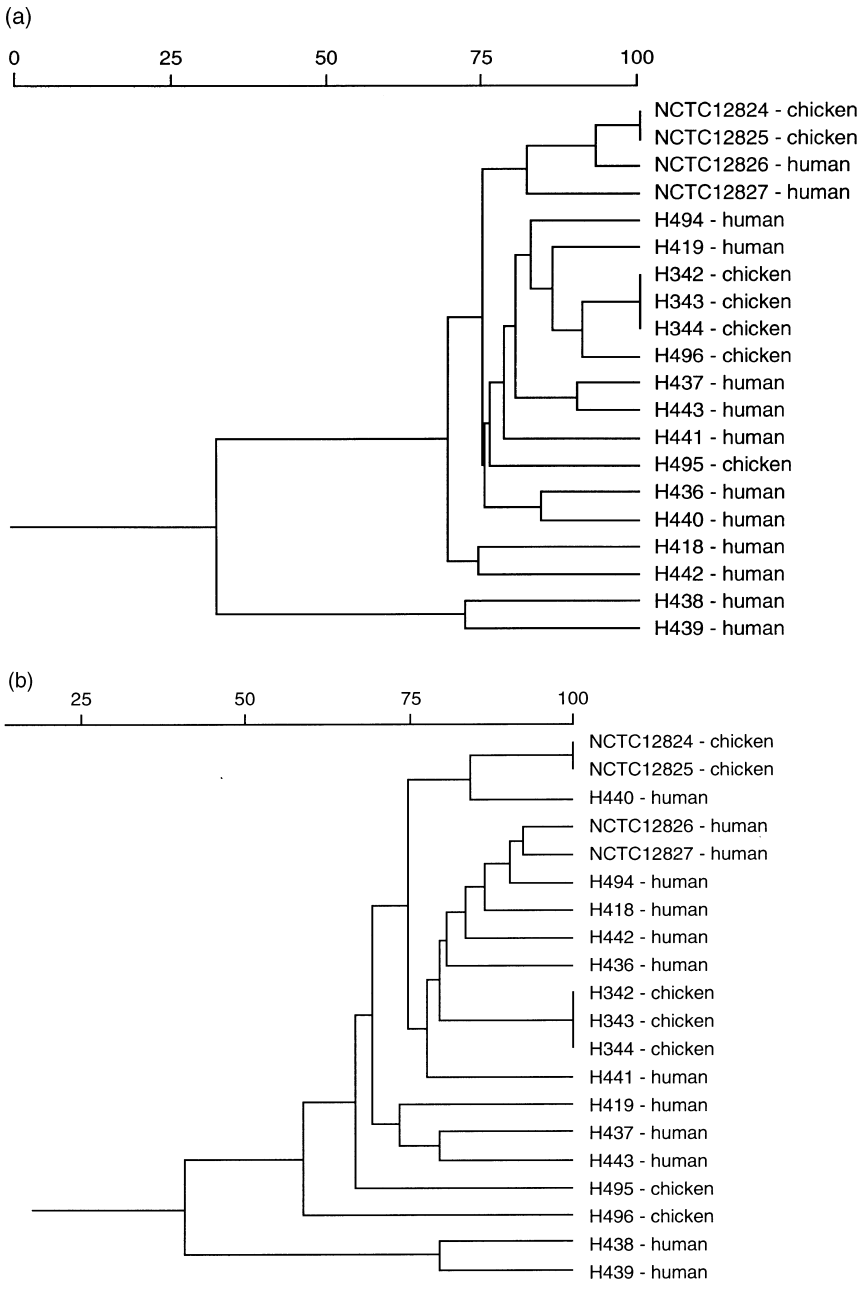

Fig. 2 Dendrograms resulting from the analysis of *Helicobacter pullorum* AFLP profiles generated by (a) primer HI-C and (b) primer HI-G. Strain numbers and hosts are shown on the vertical axis. The numbers on the horizontal axis indicate the percentage similarities as determined by the Dice correlation coefficient and UPGMA clustering

in fragment sizes was observed in the remaining *Sac*II digests (Fig. 3b) and there were no fragments that were common to all strain profiles. In general, the *Sma*I-generated banding patterns contained too many poorly resolved fragments (smaller than 97 kbp) for visual comparison (Fig. 3b). On initial analysis, the digested DNA of four strains (H494, H496, H438 and H439) stained weakly with ethidium bromide after PFGE, indicating the presence of an active endogenous DNase. However, all four samples gave clearer patterns following the inclusion of the formaldehyde treatment step during agarose block preparation.

DISCUSSION

The aims of the present study were to establish genotyping methods for characterizing individual isolates of *H. pullorum*, and then to evaluate interstrain genomic diversity, in particular, to compare human isolates with those from chickens. It

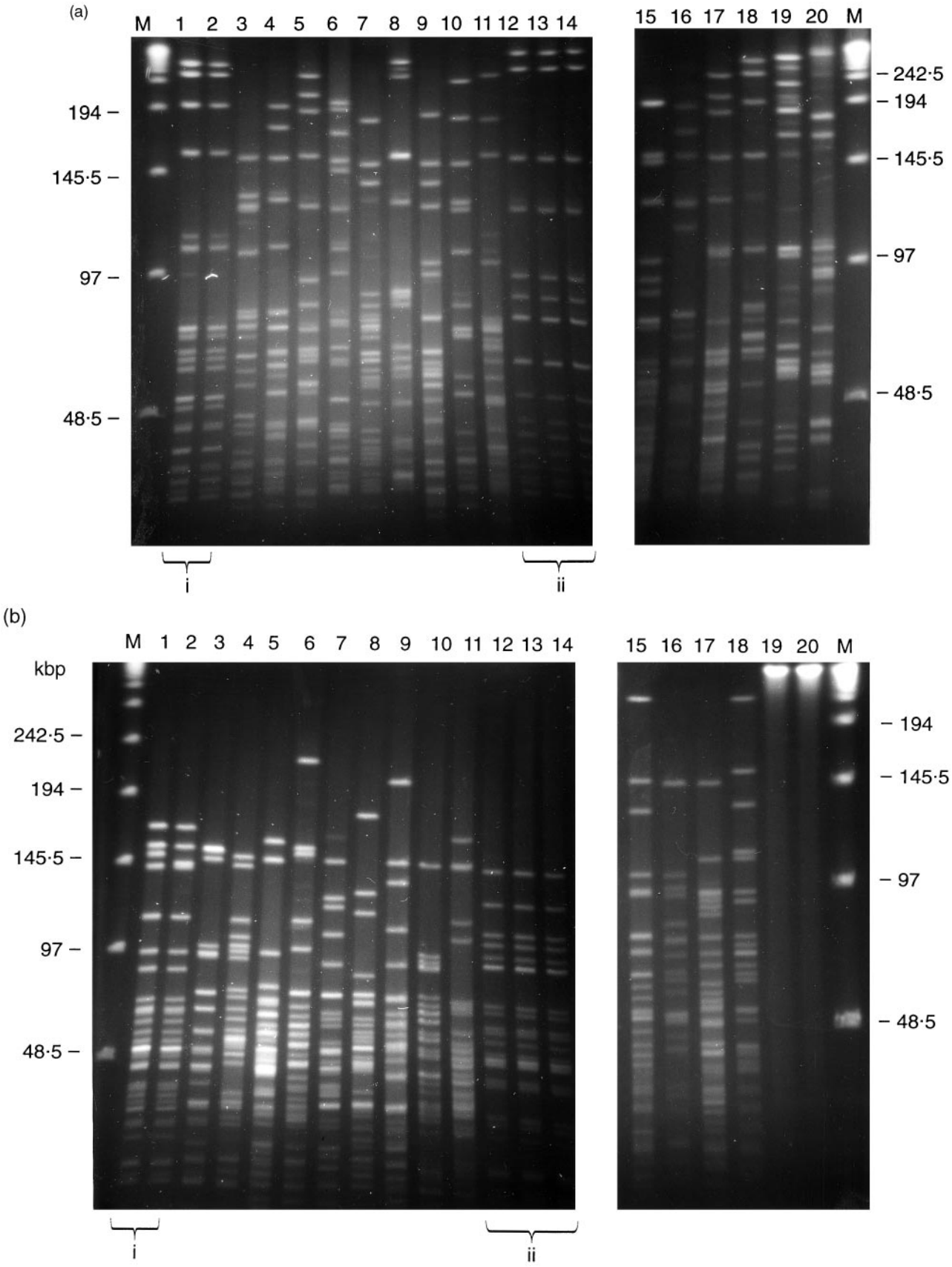

Fig. 3 PFGE of *Helicobacter pullorum* DNA digested with (a) *Sac*II and (b) *Sma*I. Lane contents are listed in Table 1. Strains bracketed are: (i) NCTC 12824 and NCTC 12825; (ii) H342, H343 and H344. Lanes marked M contain phage lambda DNA size markers (48·5 kbp concatamers; New England Biolabs)

was found that the AFLP technique developed previously for *H. pylori* (Gibson *et al*. 1998), and using *Hin*dIII digested DNA, could be directly applied to the analysis of DNA from *H. pullorum*, and that all strains could be fingerprinted. Similarly, all the strains were successfully fingerprinted by *Sac*II genomic digests. The results with both the AFLP and the PFGE analysis indicated a high degree of genomic diversity within *H. pullorum*, irrespective of the host and geographical origins of the strains. The main exceptions were the close genomic similarities observed between two of the Swiss strains (NCTC 12824 and NCTC 12825) isolated from the faeces of healthy broiler chickens from the same flock; the highly conserved polymorphisms indicated they were most probably derived from a single clonal line infecting that flock. Likewise, the three UK isolates from chicken carcasses on a factory processing line had identical genotypes and were probably from the same flock on one farm, as far as could be established from available information (personal communication, H.I. Atabay). For the other chicken isolates and all the human isolates, PFGE analysis after *Sac*II or *Sma*I digestion revealed a high level of genome diversity. The *Sac*II restriction sites, in particular, were relatively unconserved in the *H. pullorum* genome, with no fragments of the same size common to all strains. The AFLP analysis confirmed these strain differences within *H. pullorum*, although the overall level of genomic similarity (about 70%) between strains based on numerical analysis of the AFLP profiles was higher than that apparent from visual matching of the PFGE patterns. These AFLP findings confirmed the overall similarity between strains based on previous numerical analysis of total protein patterns (SDS PAGE), which showed some diversity but nevertheless clustered all strains at the $\geqslant 85\%$ similarity level (Stanley *et al*. 1994). The latter analysis included the type strain as well as several other strains examined in the present study (NCTC 12825, NCTC 12826 and NCTC 12827), and was in accord with the close similarity found between the Swiss chicken isolates NCTC 12824^{T} and NCTC 12825. A subsequent protein profiling study, which included three of the UK chicken isolates described here and the type strain, found those strains were essentially indistinguishable from each other, although the profiles were not subjected to any form of numerical analysis (Atabay *et al*. 1998).

The degree of intraspecific genome diversity observed for *H. pullorum* was comparable with that reported for *H. pylori*, which has been extensively studied by various genotyping methods (Owen and Gibson 1997), including AFLP (Gibson *et al*. 1998), because of its role as a factor in peptic ulcer disease (NIH Consensus 1994). By contrast, only minimal diversity in PFGE patterns after *Sac*II was observed amongst strains of *H. mustelae*, a species that infects the stomachs of ferrets and provides a useful model for studying pathogenesis (Taylor *et al*. 1994). PFGE after *Sma*I digestion has also been applied to determine genomic diversity in *H. hepaticus*, a species associated with active hepatitis in laboratory mice (Saunders *et al*. 1997). Polymorphisms were found in isolates from several sources within the US and three European countries. It was suggested that the level of genomic diversity within *H. hepaticus* was less than that of *H. pylori* but more than *H. mustelae*. The present results on *H. pullorum* would indicate that this species was more diverse than *H. hepaticus* (Saunders *et al*. 1997) but perhaps less so than *H. pylori* (Taylor *et al*. 1992), although comparable PFGE profile data on that species are limited, possibly because of problems caused by endogenous DNAse activity (unpublished observations). One of the most striking features of the numerical analyses of the AFLP profiles of *H. pullorum* was the fact that two human strains (H438 and H439) were similar to each other but more distantly related (linked at the 30–40% similarity level) to the rest of the species members. These isolates were both from human infections in Canada and were distinct from the other Canadian isolates as well as from human isolates from other countries. These two strains were also uniquely different from species members in their *Sac*II PFGE profiles (not digested by *Sma*I), although they were confirmed as members of *H. pullorum* by the *H. pullorum* species-specific PCR assay.

Helicobacter pullorum has been implicated in gastrointestinal disease, although a causal role as suggested by Burnens *et al*. (1994) remains unproved. However, it has been suggested by several investigators (Steinbrueckner *et al*. 1997; Atabay *et al*. 1998) that problems with isolation and species identification may have led to the underestimation of the prevalence of human disease caused by *H. pullorum*. In common with other helicobacters and campylobacters, *H. pullorum* is inert in most conventional biochemical tests used commonly in routine phenotypic identification, and it is possible that faecal isolates may have been misidentified in the past. For instance, the species cannot be distinguished from *Camp. coli* except by its lack of ability to hydrolyse indoxyl acetate, and is identical to *Camp. lari* in all respects except for its lack of tolerance to 2% NaCl and sensitivity to nalidixic acid. These features and the unsheathed flagellum would suggest *H. pullorum* had close affinities to species of *Campylobacter*. However, that is not supported by several independent phylogenetic analyses showing its relatedness to other species of *Helicobacter*, in particular to *H. rodentium,* a novel urease-negative species with non-sheathed flagella isolated from laboratory mice (Shen *et al*. 1997). It was therefore important in the present study to use the *H. pullorum* species-specific PCR assay based on the 16S rRNA gene (Stanley *et al*. 1994) to confirm the correct identity of the strains examined. *Helicobacter pullorum* is reported to be DNAse-negative (Atabay *et al*. 1998) but in the present study, after PFGE analysis of four of the strains, low concentrations of DNA were found in the agarose gel after staining. That suggested endogenous DNAse activity, even

though these strains were negative in the conventional test for DNAse activity.

AFLP is a relatively new addition to the range of techniques available for investigating microbial genomes for strain molecular typing and fingerprinting, whereas PFGE is well established and has been successfully applied to epidemiological studies of many bacterial species, including the *Helicobacter* species discussed above, and to *Camp. jejuni* and *Camp. coli* (Gibson *et al*. 1995; Owen and Gibson. 1997). This is the first study describing the use of PFGE and AFLP for genotyping of *H. pullorum*. It was evident from the results that both methods were applicable to all strains, provided equivalent levels of discrimination, and was reproducible as demonstrated by the identical fragment patterns obtained for some of the UK and Swiss chicken isolates. In conclusion, the AFLP analysis was significantly faster to perform than PFGE but in the future, both should prove to be valuable tools for improving our understanding of the epidemiology of this emerging new pathogen and its possible significance in food-borne disease.

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