FIGURE 1: Pulsed-field gel electrophoresis (PFGE) patterns of representative STEC O157 isolates from bovine samples at slaughter. UPGMA dendrogram was constructed with the use of FingerPrinting II software.

The PFGE types are known to be altered during passage in bovine intestine [17], and therefore the identical PFGE patterns of O157 isolates from offal and feces of bovines at slaughter suggest that this dissemination of O157 might be due to animal-to-animal contact at preslaughter (including farm environment) and/or cross-contamination at slaughter, respectively. In this relation, Arthur et al. (2011) reported that the O157 cells could survive on cattle hides for up to 9 days after infection [18], suggesting that animal-to-animal contact at preslaughter might be also one of the important factors for the bacterial dissemination. In addition, the detection of STEC O157 at the surface of tongues and omasum further provided an idea that this pathogen could be disseminated at slaughter by diapedesis of oral and gastric contents to the surroundings. In this support, Bergholz and Whittam reported the superior ability of STEC O157 in acid resistance to the other serotypes of STEC [19].

4. Conclusion

Our data showed the prevalence of STEC O157 in bovine feces and offal at slaughter in Japan. The genetic characterization reposes the importance for the proper salinity control at bovine (pre)slaughtering processes to prevent spread of STEC O157 to beef carcasses and offal.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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ORIGINAL ARTICLE

Continued widespread dissemination and increased poultry host fitness of *Campylobacter jejuni* ST-4526 and ST-4253 in Japan

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Abstract

Aims: *Campylobacter jejuni* is a major cause of foodborne gastroenteritis. We previously reported the widespread *Camp. jejuni* sequence type (ST)-4526 in Japan from 2005 to 2006. This study assesses the potential for this genotype to thrive thereafter.

Methods and Results: Fifty human *Camp. jejuni* isolates collected in 2010–2011 in Osaka, Japan, were genotyped by multilocus sequence typing (MLST). This approach identified 22 STs and 11 clonal complexes (CCs), including four novel STs. A comparative analysis to the previous data set showed the predominance of CC-21, in which ST-4526 and ST-4253 represented 39 and 63% in each of the two time frames, indicating their continued widespread presence. These two STs belong to close evolutionary lineages and are also isolated from chicken meat. The superior abilities of ST-4526/ST-4253 representatives to colonize chicken gut were demonstrated by co-infections with ST-21, ST-50 and ST-8 representatives.

Conclusions: Data provide evidence for the continued widespread of ST-4526/ST-4253 among human clinical isolates in Japan. These STs showed adaptive fitness to chicken.

Significance and Impact of the study: This is the first evidence of the continued thriving of ST-4526/ST-4253 in Japan with their increased *in vivo* fitness. Our findings suggest that poultry mediates the microevolution of this pathogen, thereby enabling these STs to become widespread.

Introduction

Campylobacter jejuni is one of the leading causes of food-borne gastroenteritis in humans worldwide (Friedman *et al.* 2000). This Gram-negative, microaerophilic bacterium is widely distributed in a variety of host and hostile environments, of which poultry and cattle are considered to be major reservoirs for human infection (Sanad *et al.* 2011; Hermans *et al.* 2012). The sanitary control of these habitats is therefore likely to contribute to the prevention of human campylobacteriosis. However, progress on this front has been limited, partly because of our limited understanding of how this pathogen is disseminated and which bacterial populations are most frequently associated with human infection.

This pathogen shows high genomic plasticity (Lefébure *et al.* 2010; Sheppard *et al.* 2011), which could be mediated through horizontal gene transfer (Avrain *et al.* 2004), natural transformation (Vegge *et al.* 2012), conjugative transfer (Oyarzabal *et al.* 2007) and bacteriophage predation (Scott *et al.* 2007). Many efforts focussing on this genomic diversity have attempted to genotype *Camp. jejuni* isolates from different sources and to trace the sources or routes of human transmission. The methods used have included amplified fragment length polymorphism (AFLP), *flaA* short variable region (SVR) sequencing, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (Ahmed *et al.* 2012). The MLST approach, which relies on the nucleotide sequence variation of seven *Camp. jejuni* housekeeping

genes, has been one of the most powerful genetic tools for revealing the population structures of this pathogen in many industrialized countries (Dingle *et al.* 2001; Sails *et al.* 2003; Lévesque *et al.* 2008; Korczak *et al.* 2009).

We recently used the MLST approach to report the population genetics of *Camp. jejuni* isolated from humans, poultry and cattle in Japan from 2005 to 2006 (Asakura *et al.* 2012). Comparative genome analyses further provided possible explanations for the wide distribution of ST-4526 in both human and poultry populations (e.g. increased cell adhesion, decreased DNA uptake/recombination and fluoroquinolone/nalidixic acid resistances) (Asakura *et al.* 2012). However, it was unclear whether this and the related genotype(s) might remain widespread continued in Japan after years 2005–2006, especially among the human population.

In this study, we collected MLST data for human clinical isolates from 2010 to 2011, most of which had associated epidemiological data about the infected individuals (e.g. causative foods). Comparative population genetic analysis between the two time frames revealed that ST-4526 and the related ST-4253 continued to be widespread among the human population in Japan. Through phenotypic and macrogenotypic analyses (serotype, antibiotic resistance, pulsed-field gel electrophoresis patterns and chicken infection assays), we demonstrated the close evolutionary lineage of and increased poultry host adaptation to ST-4253 and ST-4526. Finally, we discussed how these genotypes established such a thriving population in Japan.

Materials and methods

Bacterial strains and media

Human clinical isolates of *Campylobacter jejuni* ($n = 50$, designated as H_0101 to H_0150) were randomly selected from 111 *Camp. jejuni* isolates originated from a total of 46 foodborne infections in Osaka prefecture, Japan, between 2010 and 2011. These isolates were obtained from the patient's faecal samples and were biochemically confirmed to be *Camp. jejuni* according to the ISO10272 method (International Organization for Standardization 2006). The bacteria were routinely grown on Mueller-Hinton (MH) agar or in MH broth (BD Bioscience, Franklin lakes, NJ, USA) at 37°C under humidified microaerophilic conditions using an AnaeroPack-Microaero gas system (Mitsubishi Gas Chemicals, Tokyo, Japan).

Multilocus sequence typing (MLST) analysis

The bacterial DNA was extracted with a DNA tissue extraction kit (Qiagen, Hilden, Germany) and subjected to MLST analysis as described previously (Asakura *et al.* 2012). The sequences obtained were aligned with CLC DNA workbench software equipped with the MLST module (CLC Bio, Aarhus, Denmark) according to the manufacturer's instructions. The MLST profiles were deposited into the *Campylobacter* MLST database (http://www.pubmlst.org/campylobacter) to ascertain sequence types (STs)/clonal complexes (CCs) and to obtain isolate IDs according to the guidelines provided by the website. The seven-locus allele numbers were then used to construct the minimum spanning tree (http://pubmlst.org/perl/mlstanalyse/mlstanalyse.pl?site=pubmlst&page=mst&referrer=pubmlst.org). The F_{ST} fixation index of each CC was calculated by the use of the 3309-bp concatenated sequences with DnaSP ver. 5.10.1 program (Rozas *et al.* 2003) accordingly to estimate the gene flow between the two time frames (2005–2006 *vs* 2010–2011).

Antibiotic susceptibility test, serotyping and PCR detection of *tetO* gene

Disk diffusion tests using Sensi-Disk (BD Bioscience) were performed for the antibiotics norfloxacin (NFLX, 10 μg), ofloxacin (OFLX, 5 μg), ciprofloxacin (CPFX, 5 μg), nalidixic acid (NA, 30 μg), erythromycin (EM, 15 μg), tetracycline (TC, 30 μg) and gentamicin (GM, 10 μg) according to the manufacture's instruction. The PCR detection of the *tetO* gene from total and plasmid DNA prepared with Wizard plus SV minipreps DNA purification systems (Promega, Fitchburg, WI, USA) was performed as previously described (Ng *et al.* 2001). Penner serotyping was performed as described (Penner and Hennessy 1980) with a commercially available set of antisera (Denka-Seiken Co. Ltd., Tokyo, Japan). Lior serotyping was performed as described previously (Woodward and Rodgers 2002).

Pulsed-field gel electrophoresis (PFGE)

PFGE analysis was performed for all ST-4526 ($n = 4$) and ST-4253 isolates ($n = 6$) in the data set from 2010 to 2011 using the CHEF Mapper system (Bio-Rad Laboratories, Hercules, CA, USA) as described (Asakura *et al.* 2012). The obtained gel images were scanned to construct unweighted pair groups (UPGMA) with arithmetic averages using the FingerPrinting II software (Bio-Rad Laboratories).

Chicken colonization assays

The kanamycin (KM)-resistant GFP expression plasmid pWM1007 (Miller *et al.* 2000) was introduced into the isolates H_0102 (ST-4253) and H_0105 (ST-4526) by

electroporation as previously described (Asakura *et al.* 2007), and these strains were then designated as H_0102_{KM} or H_0105_{KM}, respectively. Two-week-old white leghorns free of specific pathogens ($n = 5$ per group) obtained from Nisseiken, Tokyo, Japan, were infected orally with approximately 8 $\log_{10}$ of *C. jejuni* cells in mixed cultures, consisting of almost equal numbers of H_0102_{KM} (ST-4253), H_0112 (ST-21), H_0126 (ST-50) and H_0106 (ST-53) (for group I) or of H_0105_{KM} (ST-4526), H_0112 (ST-21), H_0126 (ST-50) and H_0106 (ST-53) (group II). The H_0112 (ST21), H_0126 (ST-50) and H_0106 (ST-53) isolates were confirmed to be sensitive to KM and other antimicrobials tested in this study (see Table S1 for more detailed information). The two groups were separately fed *ad libitum*. At 1 and 2 weeks postinfection (*p.i.*), the animals were sacrificed, and their caeca were aseptically removed, weighed and homogenized in ninth volumes of sterile phosphate-buffered saline (PBS) (Life technologies, Carlsbad, CA, USA). The bacterial suspension and its serial dilutions were then plated onto mCCDA agars (Oxoid, Hampshire, UK) supplemented with or without KM (200 μg ml^{-1}) to count both KM-resistant and total *Camp. jejuni* (we confirmed that all isolates except H_0102_{KM} and H_0102_{KM} were sensitive to KM). Simultaneously, the agar plates without KM were fluorescently scanned using a Molecular Imager Fx (Bio-Rad Laboratories) to estimate the per cent of the population that was composed of GFP-positive cells. Twenty colonies were randomly selected from the KM-supplemented agar and subjected to MLST profiling to confirm isogenies during the *in vivo* colonization. The above animal experiments were performed according to the guidelines for animal care and use in National Institute of Health Sciences, Japan.

In vitro growth assay

Cells from the isolates used in the above *in vivo* infection assays ($1.0–1.4 \times 10^5$) were independently grown in MH broth at 37°C with shaking (150 rpm) under microaerophilic conditions. The turbidity of the cultures was measured chronometrically at an optical density of 600 nm at 24-h intervals for up to 72 h after incubation. Simultaneously, the same numbers of each isolate were co-cultured as groups I and II and then incubated in MH broth for 72 h, at which point 50-μl aliquots were incubated in 10 ml of fresh MH broth for an additional 72 h (we confirmed that these incubation periods were sufficient for reaching turbidity to plateau ranging from 1.3 to 1.5 of OD$_{595}$). After the repeated passaging of these cells for a total of 12 days, the growth of the pWM1007-harbouring isolates (H_0102_{KM} or H_0105_{KM}) was

measured as a percentage of the population (by plating the co-culture onto MH agars followed by fluorescent scanning as described above).

Statistics

Significant differences for the *in vivo* colonization or the *in vitro* growth fitness of ST-4526/4253 representatives were calculated using Student's *t*-test for at least three independent assays.

Results

Population dynamics of *Campylobacter jejuni* human isolates in Osaka, Japan between the periods 2005–06 and 2010–11

In this study, we collected 50 representative human clinical isolates in Osaka, Japan, in 2010-2011 (H_0101 to H_0150), which were used to analyse *Camp. jejuni* population genetics based on MLST. We identified that 44 of which were associated with nine clonal complexes (CCs). There were six singletons, including three novel sequence types (ST) (Tables 1 and S1). CC-21 was present in 36.4% (16/44), CC-22 was present in 27.3%, and CC-42/443/574/61 were present in 6.8% of the isolates, respectively (Fig. 1a). A comparison with our previous data set ($n = 50$, H_0051 to H_0100, which were human clinical isolates in Osaka, in 2005–2006; see Asakura *et al.* 2012 for more details) showed that CC-21 remained the most prominent clonal complex (Fig. 1a). The abundance of CC-22 increased from 12.0% (of the 2005–2006 data set), whereas CC-48 decreased from 14.0% (Fig. 1a). The F_{ST} values indicated no significant population differences in these CCs between the two time periods (Table 1). Within CC-21, ST-4526 ($n = 4$) and ST-4253 ($n = 6$) were predominant (Fig. 1b). The serotype analysis showed that all of the ST-4526/ST-4523 isolates were serotyped to O:2, and this serotype was restricted to within CC-21 (Tables 1 and S1). The second most abundant clonal complex, CC-22, included 11 ST-22 isolates (22%), 9 of which serotyped to O:19 (Tables 1 and S1). Together, these data indicate that ST-4526 has been continuously distributed in the human population in Osaka, Japan, between 2005–2006 and 2010–2011.

ST-4526 and ST-4253 stably acquire antimicrobial resistance

There is mounting evidence that the acquisition of certain antibiotic resistance increases the fitness of this pathogen and potentially enhances its ability to colonize hosts (Luo *et al.* 2005; Lin and Martinez 2006). In particular,

Table 1 Summary of *Campylobacter jejuni* isolates in Osaka, Japan, in 2005–2006 and 2010–2011

CC (% total; F_{ST}*)	ST†	2005–2006				2010–2011			
		No. isolate	% resistance‡		Dominant serotype§	No. isolate	% resistance‡		Dominant serotype
			NA/FQs	TC			NA/FQs	TC	
CC-21 (34%; 0·02696)		18				16			
	ST-4526	6	100	100	O: 2 (6)	4	100	100	O: 2 (4)
	ST-4253	1	100	0	O: 2 (1)	6	100	0	O: 2 (6)
	ST-50	5	40	60	O: 2 (3)	2	50	50	O: 2 (2)
	ST-21	3	67	33	O: 2 (3)	1	0	0	O: 2 (1)
	ST-451	1	0	0	O: 2 (1)	–	–	–	O: 2 (1)
	ST-8	1	0	100	O: 2 (1)	–	–	–	O: 2 (1)
	ST-4614	1	0	0	O: 2 (1)	–	–	–	O: 2 (1)
	ST-53	–	–	–	–	2	0	0	–
	ST-293	–	–	–	–	1	100	100	–
CC-22 (18%; 0·00022)		6				12			
	ST-22	6	17	0	O:19 (5)	11	91	27	O:19 (9)
	ST-5800	–	–	–	–	1	100	0	UT (1)
CC-443 (9%; 0·00186)		3				3			
	ST-5799	–	–	–		1	0	10	0:37 (1)
	ST-51	1	100	100	O: 37 (1)	1	100	100	O: 37 (1)
	ST-440	2	0	50	O: 37 (2)	1	100	100	O: 37 (1)
CC-48 (8%)		7				1			
	ST-48	4	0	25	O: 4 (2)	1	0	0	O: 4 (1)
	ST-38	1		10	UT (1)	–	–	–	
	ST-453	1	0	10	UT (1)	–	–	–	
	ST-918	1	100	0	UT (1)	–	–	–	
CC-42 (8%; 0·00029)		5				3			
	ST-42	4	0	0	O: 23 (2)	3	0	33	O: 23 (2)
	ST-447	1	0	0		–	–	–	
CC-45 (4%; 0)		2				2			
	ST-45	2	50	50	O: 12(1)/55 (1)	2	50	50	O: 12(1)/55(1)
CC-574 (3%)		0				3			
	ST-305	0	–	–	–	2	100	100	O: 5 (1)
	ST-2031	0	–	–	–	1	100	0	UT (1)
CC-61 (3%)		0				3			
	ST-61	–	–	–	–	3	0	0	O: 4 (2)
CC-52 (1%)		1				0			
	ST-52	1	100	100		–	–	–	–
CC-354 (1%)		1				0			
	ST-354	1	0	0		–	–	–	–
CC-257 (1%)		0				1			
	ST-257	–	–	–	–	1	100	100	O: 11 (1)
Unassigned (13%, 0·08306)		7				6			
	ST-2328	1	0	0		3	33	100	UT (3)
	ST-922	3	0	0		1	0	100	O: 3 (1)
	ST-5801	–	–	–	–	1	100	0	UT (1)
	ST-5802	–	–	–	–	1	0	100	O: 18 (1)
	ST-407	2	50	50		–	–	–	–
	ST-4390	1	0	100		–	–	–	–
Total		50	36	36		50	64	42	

Frequency of CCs/STs detected in two human clinical data sets obtained in 2005–2006 and 2010–2011 in Japan.

*F_{ST} values were calculated in each CC that contained >2 isolates between the two time frames.

†Novel STs are shown in bold.

‡% resistance of antimicrobials are shown per STs.

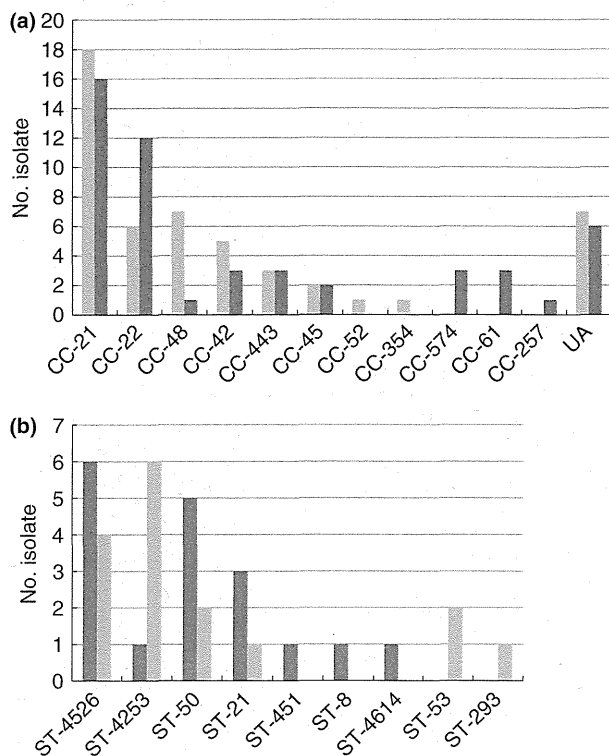§UT represents untypeable. No. of isolates is shown in parentheses.

Figure 1 Population dynamics of human *Campylobacter jejuni* isolates in Japan over time. (a) Frequency distribution of the *Camp. jejuni* clonal complexes (CCs) isolated from humans in Osaka, Japan, in 2005–2006 (open bars) and 2010–2011 (closed bars) (*n* = 50 each), which were classified into 11 clonal complexes (CCs). *UA represents isolates unassigned to any CC. (b) Frequency of sequence types (STs) within CC-21 is shown in panel (a). Novel STs are shown in bold. (▨) 2005–2006; (▦) 2010–2011.

and ST-4253 led us to focus on their evolutionary lineages.

Evolutionary lineage and macrogenotype of ST-4526 and ST-4253

As ST-4526 and ST-4253 are both widespread, their evolutionary relatedness was investigated using minimum spanning tree analysis based on the MLST allele profiles. This revealed the close lineage of the two STs within our combined data set for the periods 2005–2006 and 2010–2011 (Fig. 2a). To further examine the genetic variety within the ST-4526 and ST-4253 isolates, pulsed-field gel electrophoresis (PFGE) analysis was also conducted on the ST-4526 (*n* = 4) and ST-4253 isolates (*n* = 6) collected in this study. This analysis indicated that all of the ST-4526 isolates exhibited identical PFGE patterns, whereas six ST-4253 isolates exhibited strain-dependent variations (Fig. 2b). Thus, these data indicate that there is higher genomic similarity within ST-4526 than within ST-4253.

Increased fitness of ST-4526 and ST-4253 in the chicken gut

An epidemiological survey (questionnaire by healthcare centres at an average of 5·4 days after infection, which ranged from 1 to 12 days of periods, data not shown) revealed that the infected individuals from whom all of the ST-4526 and ST-4253 strains were isolated ate raw or undercooked chicken meat (Table S1). Considering the high population frequency and close evolutionary lineage of ST-4526 and ST-4253, we hypothesized that the poultry host might amplify those STs in the gut, thereby mediating their dissemination. To confirm this idea, we examined the colonization fitness of the representative isolates of the two STs (H_0102$_{KM}$ (ST-4253) and H_0105$_{KM}$ (ST-4526), in which the KM resistance plasmid pWM1007 was introduced) in chicken intestines by co-infecting the birds with other representative CC-21 isolates, including H_0112 (ST-21), H_0126 (ST-50) and H_0106 (ST-53) (which were sensitive to KM/FQs/NA/TC, data not shown). As shown in Fig. 3a, at 1 week *p.i.*, plate counts showed that the KM-resistant population made up 41% or 27% of the total number of *Camp. jejuni* (1·25 ± 0·34 × 10^7 of 3·05 ± 1·02 × 10^7 CFU g^{-1}, or 1·05 ± 0·32 × 10^7 of 3·94 ± 0·81 × 10^7 CFU g^{-1}), and this increased to 74% or 78% (4·79 ± 0·96 × 10^7 of 6·49 ± 1·58 × 10^7 CFU g^{-1}, or 7·46 ± 0·83 × 10^7 of 9·52 ± 1·34 × 10^7 CFU g^{-1}) at 2 weeks *p.i.* in group I (H_0102$_{KM}$+other) and group II (H_0105$_{KM}$+others), respectively (Fig. 3a). Despite the difference in absolute colonization levels between the two groups, in both

fluoroquinolone (FQ) resistance is a matter of concern in the public health field (Perez-Boto *et al.* 2012). We therefore characterized the antimicrobial resistances of the human clinical isolates with a disk diffusion test. Among them, 12 of 16 CC-21 isolates (75%) exhibited resistance to nalidixic acid (NA) and FQs, and all of the ST-4526 and ST-4253 isolates were included in this group (Table 1). All of the ST-4526 isolates also exhibited tetracycline (TC) resistance. 10 of 11 ST-22 isolates that were consisted of a total of 12 CC-22 isolates also exhibited resistance to NA and FQs, whereas all of the isolates of CC-42 (*n* = 5) and CC-61 (*n* = 6) were susceptible to all of the antimicrobials in the data set from 2005 to 2006 (Table 1). Similar trends were observed in the data set on 2005–2006 (Asakura *et al.* 2012), except for relatively high yield of NA/FQs-susceptible ST-22 isolates (five of six isolates, Table 1). The differences in TC resistance between ST-4526 and ST-4253 corresponded to the presence of the plasmid-mediated *tetO* gene (Fig. S1). The frequent antimicrobial resistance observed in ST-4526
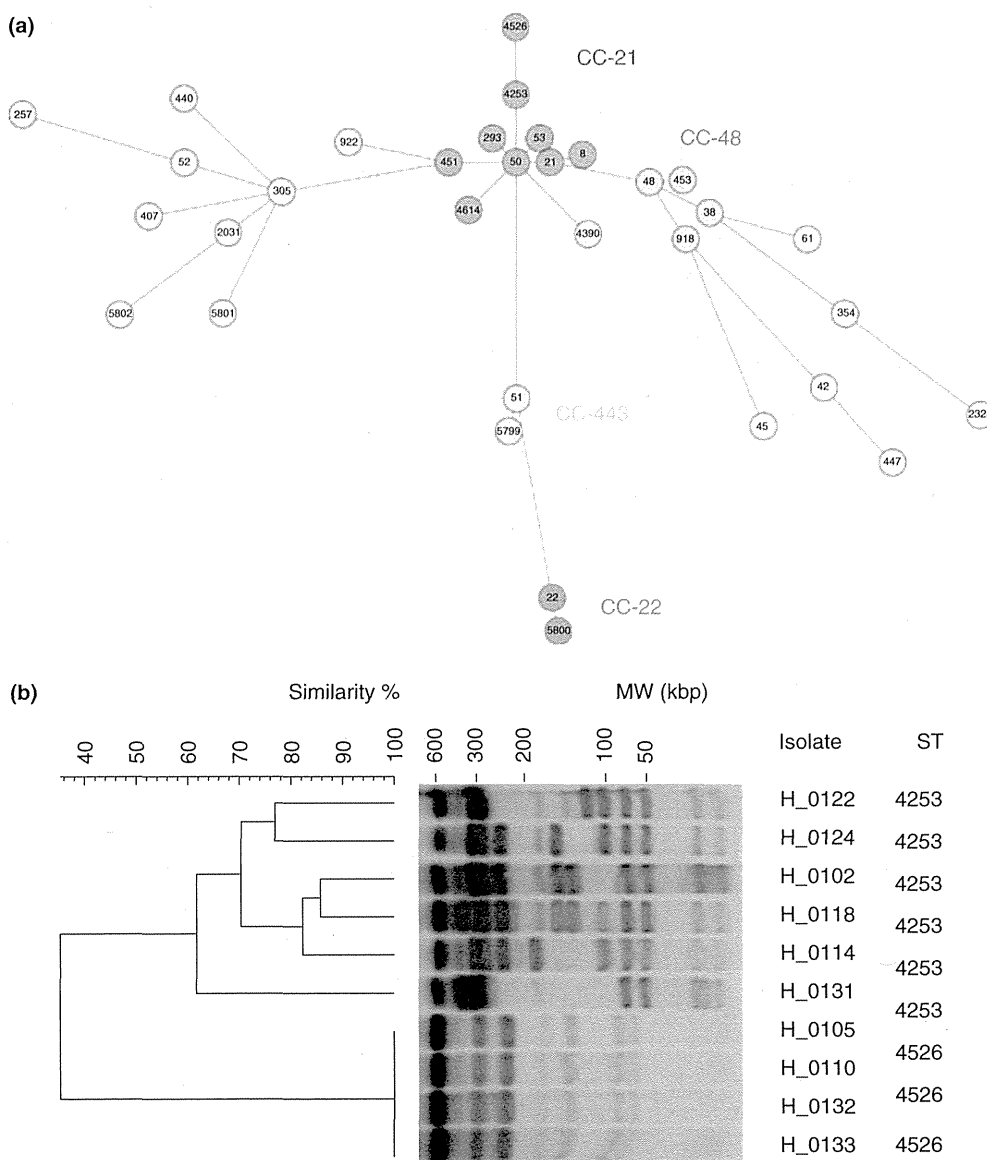
Figure 2 Phylogenetic analyses of human *Campylobacter jejuni* isolates in Japan. (a) Minimum spanning tree of *Camp. jejuni* MLST profiles from the combined data sets for 2005–2006 and 2010–2011. ST numbers are shown in the circles. (b) Pulsed-field gel electrophoresis (PFGE) analysis of four ST-4526 and six ST-4253 isolates in 2010–2011 and two ST-4526 isolates (H_0060 and H_0097) in 2005–2006. Samples were *Sma*I-digested and loaded onto a 1% agarose gel. The UPGMA dendrogram divided these isolates into four groups, with a cut-off value of 70% similarity.

experiments the fraction of GFP-expressing bacteria (ST-2453 in group 1 and ST-4526 in group II) increased from 25% in the inoculum to over 70% 2 weeks *p.i.* (Fig. 3a). Fluorescent scanning also showed an increase in the percentage of the GFP-positive population with no significant differences between groups I and II (Fig. 3a). Despite the increased *in vivo* fitness of the two representatives assayed, all of the tested isolates showed similar growth rates [without significant differences (Fig. 3b)], and the H_0102$_{KM}$ (ST-4253) and H_0105$_{KM}$ (ST-4526)

isolates did not significantly out-compete the others under *in vitro* co-culture conditions (Fig. 3c). Together, these results demonstrated that the ST-4526/ST-4253 representatives showed increased fitness over time in the chicken gut.

Discussion

We analysed the population genetics, antimicrobial resistance and serotypes of 50 human clinical *Camp. jejuni*

Figure 3 ST-4526 and ST-4253 representatives exhibit increased chicken colonization and transmission. (a) Results of two independent colonization experiments, in which two groups of chickens were colonized with a mixture of four *Campylobacter* strains. The mixture included either 25% bacteria of a ST-4253 strain (Group I) or of an ST-4526 strain (Group II) that contained a plasmid conferring KM resistance as well as GFP expression. Colonization levels obtained after 1 and 2 week *p.i.* are shown as absolute counts (total counts in black, KM-resistant counts in white) and as relative proportion of GFP-expressing bacteria (grey). (b) *In vitro* growth kinetics of each isolate were determined by turbidity assays (OD 600 nm) at 24, 48 and 72 h incubation periods. (c) The extent of growth for H_0102$_{KM}$ (ST-4253) or H_0105$_{KM}$ (ST-4526) during co-incubation in MH broth. Percentage population of GFP-positive cells in each groups (dotted and open bars represent Group I and II, respectively) at every 72 h (3 days) postincubation are shown. (—◆—) H_0102KM (ST-4253); (—△—) H_0105KM (ST-4526); (—□—) H_0112 (ST-21); (—▲—) H_0126 (ST-50); (—○—) H_0106 (ST-53).
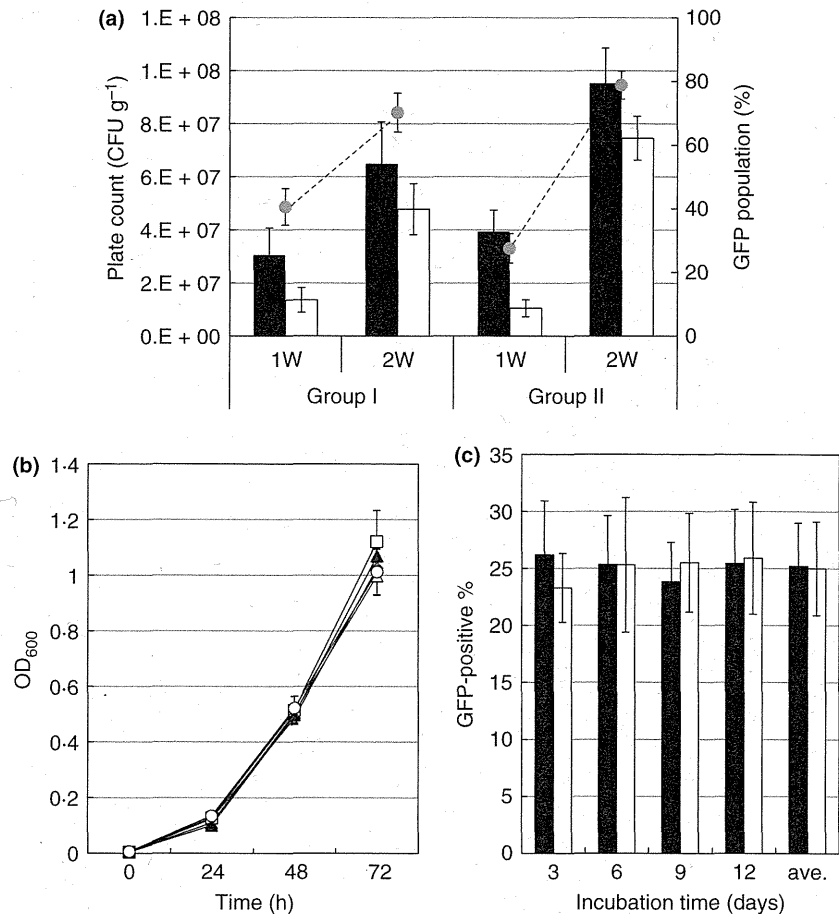
isolates collected from 2010 to 2011 in Osaka, Japan. A comparison of the MLST data set to that from 2005 to 2006 confirmed that ST-4526 and ST-4253 continued to thrive. Macrogenotypic and phylogenetic analyses suggest a close evolutionary lineage between ST-4526 and ST-4253. The *in vivo* colonization assay further demonstrated that these isolates possessed superior abilities to colonize the chicken intestine, outcompeting the ST-21, ST-50 and ST-53 isolates, suggesting that the poultry host might mediate the widespread dissemination of those STs among poultry populations, thereby increasing their occurrence in human isolates from individuals with campylobacteriosis in Japan.

The widespread genotypes ST-4526/ST-4253 showed resistance to NA/FQs. Ten of 11 ST-22 isolates also exhibited resistance to these antimicrobials. Considering that these 3 STs made up 40% of the new MLST collection (20 of 50 isolates), it is likely that this sort of anti-microbial resistance could contribute to the continuous wide dissemination of these STs in Japan. Previous work reporting the enhanced *in vivo* fitness of FQ-resistant *Camp. jejuni* to antibiotic selection pressure (Luo *et al.* 2005) supports this idea.

In previous work on serotype distribution, Saito *et al.* (2005) reported that the serotype O:2 and O:4 complex was frequently distributed in human, poultry and bovine isolates, while serotypes O: 23, 36, 53 were common in human and bovine isolates in Japan. More recently, Harada *et al.* (2009) demonstrated the predominance (32·6%) of these serotypes among 601 cattle and poultry isolates. Our data in this study further support the predominance of these serotypes and their association with CC-21 and CC-48 in human isolates in Japan. In particular, evidence for the relatively high yields of ST-4526 and ST-4253 within serotype O:2 (43·8%) of CC-21 of human isolates, which were also distributed among poultry isolates that were obtained from live birds fed at distinct area, Aichi and Yamaguchi prefectures in Japan (Asakura *et al.* 2012), further suggests that poultry might mediate the widespread of these STs in the human population in Japan.

Camp. jejuni is known to exhibit high genomic plasticity between strains (Hepworth *et al.* 2007), and this plasticity is likely to be mediated by passage through animal hosts (de Boer *et al.* 2002). A more recent report from Hänel *et al.* (2009) added evidence that passage through

the chicken gut altered PFGE patterns and *flaA*-sequences of multiple *Camp. jejuni* strains, with partial co-incidence of phenotypic alterations such as cell adhesion, motility and morphology. The differential PFGE patterns of the 6 ST-4253 isolates in this study thus indicate their *in vivo* passage backgrounds. In contrast, the identical patterns of the four ST-4526 isolates demonstrate the stable genomic features of this genotype, a finding that is also supported by comparative genomics (Asakura *et al.* 2012). Nevertheless, the *tetO* gene-mediated TC resistance of ST-4526 isolates also suggests that this genotype may acquire this feature during passage through an animal host because this gene could be horizontally transferred between *Camp. jejuni* in the chicken gut (Avrain *et al.* 2004). Thus, these results suggest that poultry is important not only as a reservoir for human infection but also as a mediator for the microevolution of this pathogen.

Given the public health concerns and commercial importance of *Camp. jejuni* as a zoonotic pathogen, it is somewhat surprising how little is known about the routes and dynamics of its colonization of chickens. Although many poultry flocks appear to be dominated by a single strain (Ring *et al.* 2005), two competing variants (Jacobs-Reitsma *et al.* 1995; Berndtson *et al.* 1996) and even greater multiplicities of strains (Thomas *et al.* 1997; Hiett *et al.* 2002) have been isolated from the same flock. Considering that this pathogen can be transmitted from bird to bird immediately after infection (Shanker *et al.* 1990), the superior colonization abilities of ST-4526 and ST-4253 in the chicken gut suggest that poultry hosts might amplify those STs, increasing the odds that they will be transmitted to humans. In addition, a recent study by Bereswill *et al.* (2012) showed that intestinal microbiota significantly modulate host innate immunity to alter *Camp. jejuni* colonization *in vivo*. As little is known about the dynamics of chicken microbiota in response to *Camp. jejuni* colonization, future work on the interactions among *Camp. jejuni*, intestinal microbiota and innate immunity in poultry hosts might improve our understanding of how this pathogen can establish longitudinal colonization in reservoirs that can then infect humans.

In summary, this study is the first to show that ST-4526 and the related ST-4253 continue to thrive in the human *Camp. jejuni* population in Japan. The results of genetic and phenotypic analyses provide possible reasons for the widespread dissemination of ST-4526 and ST-4253. Future study of the molecular basis underlying the superior host (poultry) adaptation of these sequence types will provide further insight into the link between the microevolution and ecological features of this pathogen, thereby helping to prevent transmission to humans.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Presence or absence of the plasmid-mediated *tetO* gene in ST-4526 ($n = 4$) and ST-4253 isolates ($n = 6$). PCR detection of the *tetO* gene was conducted using the plasmid DNA from *Campylobacter jejuni* ST-4526 and ST-4253 isolates. M represents 100 bp-molecular marker.

Table S1 *Campylobacter jejuni* isolates used in this study.