平成２４－２６年度厚生労働科学研究費補助金
食品の安全確保推進研究事業
と畜・食鳥検査における疾病診断の標準化とカンピロバクター等の制御に関する研究
分担総合研究報告書

と畜・食鳥検査における疾病診断の標準化に関する研究

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研究要旨：

と畜・食鳥検査における疾病診断の標準化に関する研究

食肉衛生検査は、疾病排除を主体として、食中毒予防のための食肉の衛生管理などが含まれている。その中でも、疾病廃除のための診断技術を平準化することにより、全国で同一レベルの食肉検査を行うことが可能となることが期待される。これまで、と畜・食鳥検査における疾病診断は食肉検査マニュアルを改訂することにより、診断に関する基本的事項にについては示している。しかしながら、全国のと畜検査員が同じ基準で疾病診断をする際の肉眼的診断の標準化についてどのような方法がよいかを検討する必要がある。

２４年度は、疾病診断の標準化に役立つマニュアルのあるべき姿について検討した。また、標準化の必要があると考えられる疾病について優先順位付けを行った。豚の非定型抗酸菌症は全国食肉検査所協議会で平成２４年度の検討課題となっていることから、優先的に検討することとした。残りの５疾病（牛白血病、豚赤痢、豚丹毒（関節炎型）、萎縮性鼻炎、ヨーネ病）についてはアンケート調査を行った。その結果、平成24年度は平準化を優先的に取り組むべき疾病として、非定型抗酸菌症、牛白血病、豚赤痢、豚丹毒（関節炎型）、萎縮性鼻炎、ヨーネ病の順位となった。その他の疾病としては、高度の水腫、中皮腫などが複数のブロックから提案された。

２６年度は敗血症についてマニュアルをまとめ直した。

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A．研究目的

本研究は、食肉検査マニュアルの改訂のために改訂の必要となる疾病の優先順位付けを行うことを目的とした。

また、マニュアルのあるべき姿についても検討する。

B．研究方法

食肉検査マニュアル改訂のために疾病の優先順位をつけるために全国７ブロックの食肉衛生検査所にアンケート調査を行った。

全国食肉衛生検査所協議会では豚の非定型抗酸菌症を診断の標準化対象としていることから、優先順位の１位を豚の非定型抗酸菌とし、牛白血病、豚赤痢、豚丹毒（関節炎型）、萎縮性鼻炎、ヨーネ病について順位付けを行った。

また、食肉検査マニュアルのあるべき姿について検討した。

C．研究結果

アンケート調査の結果、２位は牛白血病、以下、豚丹毒、豚赤痢、ヨーネ病、萎縮性鼻炎の順に改訂が必要との結果となった。

その他の疾病としては、高度の水腫、中皮腫などが複数のブロックから提案された。

食肉検査マニュアルの問題点として、１．必要と思われる写真やフロー図がない、２．検査マニュアルだけで完結しない、３．診断・類症鑑別に教科書的表記が混在している、４．農場での所見と・とちく場で見られる所見が混在している、５．甚急性と慢性の記載が混在している、６．表現がと畜検査上一般的ではない、７．繰り返し同じ内容が記載されているとの指摘があった。マニュアルに必要な項目として、１．解説、２．保留基準、３．採材方法、４．類症鑑別、４．検査方法、５．判定基準、６．措置についてそれぞれの項目について要点を示し、１冊で完結させる必要がある。

平成26年度は、別添のように敗血症のマニュアルについて改訂のポイントを示した。

D．考　察

食肉衛生検査マニュアルはマニュアルというよりは教科書的にまとめられていることから、食肉衛生検査にはそのままで使いづらいところがある。改訂が必要と考えられた。

たとえは、と畜検査の作業をフロー図にまとめ、チェックシート形式にすることは、各自治体の疾病診断を平準化することに役立つものと考えられた。

E．結　論

食肉衛生検査マニュアルは現場での使用を視野に入れた改訂が必要である。

F．健康危機情報

該当無し

G．研究発表

該当なし

H．知的財産権取得状況

該当なし

と畜検査マニュアルについて

疾病の診断基準
検査所内での問題＆と畜検査マニュアルの問題

各検査所の問題点

- 検査所の検査員によって判断基準が異なる
  経験年数·人員不足·知識の伝達の不均一化
- 自治体、地域によって判断基準が異·t
  同じ農場から出荷しても、廃棄状況が異なる
  保留基準等、疾病に対する解釈が自治体によって異なる
- 機器の充実度が違うため検査方法が異なる
  検査所の規模、と畜場の規模

検査マニュアルの問題点

- 必要と思われる写真やフローがない
- 検査マニュアル一冊で完結しない
  （検査法の詳細については、〇〇を参照）
- 診断ー類症鑑別に教科書的表記が混ぞ
- 農場での所見と、と畜場でみられる所見が混在
- 甚急性と慢性の症状の表記が混在
- 表現がと畜検査上一般的でない
  （肉眼病理学的を病理学的）
- 繰り返し同じ内容が記載

理想のマニュアル像とは

- 一冊で完結
  (SOPとアトラスが盛り込まれている)
- 実際のと畜検査の中で使える
- グレーゾーンが解消され
- 読みやすい、使いやすい
  （フローチャート、チェックリスト付き）
- 初心者が見てわかりやすい

マニュアルのアウト ライン

- 解説
- 保留基準
- 採材方法
- 類症鑑別
- 検査方法
- 判定基準
- 措置

「解説」のポイント

- 情報量は減らしていない
- 似たような表現を簡略化
- 診断以降の項目で、概論的な記述を解説に盛り込んだ
- と畜検査における狭義の敗血症の図式化
- 敗血症の定義で「細菌の感染」を「病原体の感染」とした（ウイルス－真菌）

「保留基準」のポイント

- 写真を掲載した
- 症状、所見を箇条書きにした

「採材部位」のポイント

採材部位

以下の点に注意して、枠内の部位及び検査員が必要と判断した部位について採材すること。

・病変部を中心に採材すること。

・再検査にそなえて十分な量を採材すること。

・リンパ節においては、敗血症を証明できるように離れた２ヵ所以上を採材すること。

（例：内側腸骨リンパ節と浅頸リンパ節）

心臓(疣状病変部と心筋)、腎臓、肝臓、脾臓、リンパ節、筋肉

- 主に採材すべき部位を箇条書きにした
- 採材量、採材方法等も記載した

「類症鑑別」のポイント

類症鑑別

解体後検査で認められた所見	留意する疾病
疣贅性心内膜炎	豚丹毒　など
臓器、リンパ節の出血、腫脹	中毒、サルモネラ症、トキソプラズマ病　など
臓器、筋肉、脂肪の黄変	黄疸　など

- 解体後検査でよく認められる所見別に、分かりやすく表にまとめた

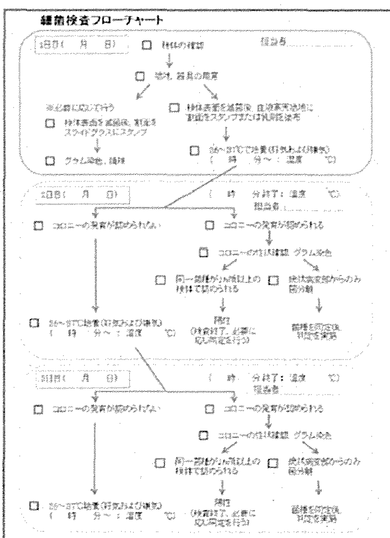
「検査方法」のポイント

検査方法

- 検査結果の写真を掲載
- 一通りの検査を行えるように準備品から記載
- レイアウトを工夫

「検査方法」のポイント

「検査方法」のポイント



- 実用的なフローチャートを掲載
- 検査経過の記載および管理が可能

「判定基準」のポイント

判定基準

(1) 生体検査時
全身性の症状を呈し、血液中に菌の存在が確認されたもの。

(2) 解体後検査時
①肉眼病理学的に敗血症を疑う所見を呈し、臓器・リンパ節・筋肉のいずれかの2ヵ所以上から同一の菌種が分離されたもの。もしくは症状病変部から病原菌が分離されたもの。

②肉眼病理学的に敗血症の一般所見を呈するもの。すなわち全身性(臓器、リンパ節、筋肉及び皮下織)の出血、主要臓器の混濁腫脹、リンパ節の腫脹、出血等多くの所見を示すもの。

*「いずれか2ヵ所」とは
・心疾症状病変部+(臓器、筋肉、リンパ節のいずれか1ヵ所)
・臓器2ヵ所
・臓器1ヵ所+筋肉
・臓器1ヵ所+リンパ節1ヵ所
・筋肉+リンパ節1ヵ所
・リンパ節2ヵ所

* 病原菌とは日本細菌学会「病原体等安全取扱・管理指針」によりバイオセーフティレベル1*以上に分類される細菌、またはその他の文献等によりヒト及び動物に対する病原性が明らかな細菌。

* 日本細菌学会「病原体等安全取扱・管理指針」によるバイオセーフティレベルの設定
(1) バイオセーフティレベル1の細菌
バイオセーフティレベル2以上の細菌を除くすべての細菌がバイオセーフティレベル1であるが、レベル1のうちで、人からの分離例があり、日和見感染症を起こす可能性のある菌種をレベル1*に分類している。

(2) バイオセーフティレベル2の細菌
健康人に感染症を起こす能力を持ち、その危険度が軽度ないし中等度であるもの。

(3) バイオセーフティレベル3の細菌
感染量が小さいため感染性が高く、重篤でしばしば致死的な感染症を起こすもの。

- 記載があいまいな表現を具体的に欄外に記載
- 基準を追加
（心疾状病変のみからの病原菌分離時）
- 文言の整理

「措置」のポイント

措置

(1) 生体検査時：と殺禁止【と畜場法第16条、施行規則第16条第1項(別表第4)】
(2) 解体後検査時：全部廃棄【と畜場法第16条、施行規則第16条第3項(別表第5の上欄)】

- 解体前検査時は判定基準がないため未記載

III.　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
Pascoe B, Lappin-Scott H, Sheppard SK, Asakura H	Does biofilm formation aid colonization and infection in *Campylobacter*?	Sheppard SK & Meric G	*Campylobacter* Ecology & Evolution	Caister Academic Press	London, UK	2014	pp. 177-188

雑誌

発表者氏名	論文タイトル名	発表誌名	巻	ページ	出版年
Asakura H, Brueggemann H, Sheppard SK, Ekawa T, Meyer TF, Yamamoto S, Igimi S.	Molecular evidence for the thriving of *Campylobacter jejuni* ST-4526 in Japan	PLoS ONE	7	e48394	2012
Asakura H, Hashii N, Uema M, Kawasaki N, Sugita-Konishi Y, Igimi S, Yamamoto S.	*Campylobacter jejuni pdxA* affects flagellum-mediated motility to alter host colonization.	PLoS ONE.	8	e70418	2013
Asakura H, Taguchi M, Ekawa T, Yamamoto S, Igimi S.	Continued widespread disseminat ion and increased poultry host fitness of *Campylobacter jejuni* ST-4526 and ST-4253 in Japan.	J Appl Microbiol.	114	1529-1538	2013
Asakura H, Masuda K, Yamamoto S, Igimi S.	Molecular approach for tracing the dissemination routes of Shiga toxin-producing *Escherichia coli* in bovine offal at slaughter.	BioMed Res Int.	2014	e39139	2014
Asakura H, Brueggemann H, Makino S, Sugita-Konishi Y.	Molecular approaches for the classification of microbial pathogens of public health significance.	BioMed Res Int.	2014	e725801	2014

Molecular Evidence for the Thriving of *Campylobacter jejuni* ST-4526 in Japan

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Abstract

Campylobacter jejuni is a leading cause of human gastroenteritis worldwide. This study aimed at a better understanding of the genetic diversity of this pathogen disseminated in Japan. We performed multilocus sequence typing (MLST) of *Campylobacter jejuni* isolated from different sources (100 human, 61 poultry, and 51 cattle isolates) in Japan between 2005 and 2006. This approach identified 62 sequence types (STs) and 19 clonal complexes (CCs), including 11 novel STs. These 62 STs were phylogenetically divided into 6 clusters, partially exhibiting host association. We identified a novel ST (ST-4526) that has never been reported in other countries; a phylogenetic analysis showed that ST-4526 and related STs showed distant lineage from the founder ST, ST-21 within CC-21. Comparative genome analysis was performed to investigate which properties could be responsible for the successful dissemination of ST-4526 in Japan. Results revealed that three representative ST-4526 isolates contained a putative island comprising the region from Cj0737 to Cj0744, which differed between the ST-4526 isolates and the reference strain NCTC11168 (ST-43/CC-21). Amino acid sequence alignment analyses showed that two of three ST-4526 isolates expressed 693aa- filamentous hemagglutination domain protein (FHA), while most of other *C. jejuni* strains whose genome were sequenced exhibited its truncation. Correspondingly, host cell binding of FHA-positive *C. jejuni* was greater than that of FHA-truncated strains, and exogenous administration of rFHA protein reduced cell adhesion of FHA-positive bacteria. Biochemical assays showed that this putative protein exhibited a dose-dependent binding affinity to heparan sulfate, indicating its adhesin activity. Moreover, ST-4526 showed increased antibiotic-resistance (nalidixic acid and fluoroquinolones) and a reduced ability for DNA uptake. Taken together, our data suggested that these combined features contributed to the clonal thriving of ST-4526 in Japan.

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Introduction

Campylobacter jejuni is one of the leading bacterial causes of foodborne gastroenteritis in humans and is responsible for an estimated 5–14% of the occurrence of human diarrhea worldwide, which translates into 400–500 million cases annually [1], [2]. This pathogen is typically located in the feces and gastrointestinal tracts of warm-blooded animals [3], [4], [5], [6], which are considered reservoirs for human infection. Human campylobacteriosis is most often sporadic; although poorly prepared food has often been implicated, it is generally difficult to determine the source since *C. jejuni* easily loses cultivability in food specimens under aerobic conditions. Therefore, a substantial effort has been made to isolate *C. jejuni* from a wide variety of reservoirs and type these strains using a number of methods, including amplified fragment length polymorphism (AFLP), *flaA* short variable region (SVR) sequencing, pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) [7]. MLST involves the phylotyping of bacterial isolates based on the DNA sequence comparison of a standardized panel of housekeeping genes [8]. As this method has advantages for accuracy and reproducibility of the data between different laboratories, MLST is one of the best approaches to understand the population genetics, host association, and seasonal or geographical variation of this pathogen [9], [10], [11], [12], [13], [14], [15], [16]. To date, 5,957 sequence types (STs) from 18,562 isolates have been obtained from MLST data, which have been deposited in the *Campylobacter* MLST database (http://www.plst.org/campylobacter) as for August 17, 2012. The clonal diversity and evolutionary linkage of the STs could be visually analysed with eBURST [17], facilitating the evolutionary analysis.

C. jejuni is a public health threat as one of the most common foodborne pathogens in Japan [18]. Results of a previous epidemiological study suggest that chicken and feedlot cattle are the main reservoirs for human campylobacteriosis [19]. Several molecular approaches, including PFGE, serotyping, and *flaA* genotyping, have provided a possible explanation for the wide-spread of this pathogen, which coincides with partial host association in Japan [20], [21], [22]. For example, serotypes O:2 and O:4 were common in human, poultry, and bovine isolates, whereas serotypes O:23, O:36, O:53 were common in human and bovine isolates in Japan [20]. The PFGE analysis also showed a partial similarity between poultry and human isolates [20]. In a

more recent study, Yabe et al. [23] conducted a small-scale MLST analysis, revealing the presence of novel STs in *C. jejuni* isolated from poultry and humans in Japan. However, it remains unclear how and whether such host associations might be linked to human campylobacteriosis and whether there are geographic specifications that contribute to the thriving of this pathogen in certain populations within Japan.

In the present study, we used an MLST approach to identify the population structure of *C. jejuni* isolated from human, poultry, and cattle across Japan for the first time. We identified a unique genotype, ST-4526, that thrived in poultry and human populations in Japan. A phylogeographical linkage analysis, in combination with comparative genome analyses, provided a possible explanation for this thriving.

Materials and Methods

Bacterial Isolates and Media

From 2005–2006, *C. jejuni* human clinical isolates (n = 100) were obtained from two prefectures, Tokyo and Osaka, in Japan. Poultry (n = 61) and cattle isolates (n = 51) were obtained from prefectures in Miyagi, Gunma, Akita, Kumamoto, Aichi, Hyogo, Yamaguchi (for poultry) or Oita and Hyogo (for cattle) during the same periods (Table S1, Fig. S1). The *C. jejuni* strain NCTC11168 [24] was used as a reference strain for comparative genomic and phenotypic studies. The *C. jejuni peb4* mutant [25] was used as a donor strain for the natural transformation efficiency assay. Bacterial isolates were grown on Mueller-Hinton (MH) agar or MH broth (Becton Dickinson, Franklin lakes, NJ, USA) at 37°C in a humidified CO_2 AnaeroPack-Microaero gas system (Mitsubishi Gas Chemicals, Tokyo, Japan).

Multi-locus Sequence Typing (MLST) Analysis

Bacterial genomic DNA was extracted using a DNeasy Genomic Extraction kit (QIAGEN, Hilden, Germany) and stored at -20°C until further use. The PCR and cycle sequencing reactions were conducted according to the guidelines on the *Campylobacter* MLST database (http://pubmlst.org/campylobacter/). We confirmed the absence of the non-specific PCR amplicon using a 1% agarose gel. ExoSAP-IT was subsequently used to purify the PCR products according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). For the sequencing reactions, we used both DNA strands in each allele with the BigDye terminator v. 3.1 Ready Reaction Cycle Sequencing kit on an ABI3730x DNA analyzer (Life technologies). The obtained sequences were assembled using CLC DNA Workbench software equipped with an MLST module (CLC bio, Aarhus, Denmark). The consensus sequences for each allele were assigned an allele number, a 7-locus (3,309 bp) sequence type (STs), and a clonal complex (CCs) through interrogation of the *Campylobacter* MLST database. Unassigned sequences were deposited in the database to obtain new allele or ST numbers according to the guidelines described on the website. The sequence data of each isolate were deposited in the database (each isolate ID is shown in Table S1).

Phylogenetic Analyses

The 7-locus allelic sequences were used to estimate the clonal genealogy of the STs using a model-based approach to determine the bacterial microevolution using ClonalFrame [26]. The output data were visualized using the iTOL (interactive Tree of Life) program (http://itol.embl.de/index.shtml) [27]. To assess clonal diversity and evolutional linkage, 7 allelic numbers for a total of 547 STs belonging to CC-21, were subjected to the Global Optimal eBURST (goeBURST, a java implementation of the eBURST algorithm proposed by Feil et al. [17]) [28].

Pulsed-Field Gel Electrophoresis (PFGE)

All ST-4526 isolates (n = 10, Tables 1 and 2) were subjected to PFGE with *Sma*I endonuclease (New England BioLabs, Ipswich, MA, USA) using the CHEF Mapper system (Bio-Rad Laboratories, Hercules, CA, USA) as previously described [29]. The gel images were obtained using ethidium bromide stain. The electrophoretic patterns from PFGE were compared based on band position using FingerPrinting II software (Bio-Rad Laboratories) and derived using the Dice coefficient with a maximum position tolerance of 1%. The strains were clustered using the unweighted pair group (UPGMA) method with arithmetic averages according to the manufacturer's instructions.

Genome Sequencing and Bioinformatics Analysis

Genomic DNA was extracted from three representative ST-4526 isolates (H_0002, H_0089, P_0053, which were selected randomly from the three different PFGE groups a, b, and c, respectively), using a Genomic tip-500 kit (QIAGEN, Hilden, Germany) and subjected to 8-kb paired-end pyrosequencing in a GS FLX+ system (Roche Diagnostics, Burgdorf, Switzerland) according to the manufacturer's instructions. The sequence data (151,327, 173,181, and 167,955 reads for H_0002, H_0089, and P_0053, respectively) were assembled using the GS *De Novo* Assembler version 2.6 (454 Life Sciences, Branford, CT, USA). 77, 63, and 54 contigs were obtained (>500 bases) for the isolates H_0002, H_0089, and P_0053 at 41, 50, and 48-fold coverages, respectively. The draft genome sequences reported in this paper have been deposited in the GenBank database with accession number DRA000568.

The detection of open-reading frames and annotation of all draft genomes were automatically performed using the RAST service pipeline [30]. The Artemis Comparison Tool (ACT; http://www.sanger.ac.uk/resources/software/act/) was used for genome-wide comparisons of the nucleotide sequences [31]. Genome comparisons were also performed using a protein sequence-based bidirectional BLAST (Bi-BLAST) approach. The Island Viewer, which is a computational tool that integrates three different genomic island prediction methods, i.e., IslandPick, IslandPath-DIMOB, and SIGI-HMM [32], was used to identify the genomic islands. The results of all Bi-BLAST comparisons are shown in Table S6.

LOS Isolation, SDS-PAGE, and Western Blot

Lipooligosaccharides (LOS) were prepared from *C. jejuni* grown on MH agar plates for 20 h at 37°C as described previously [33]. The samples were loaded onto 15% polyacrylamide gels and visualized using silver stain. The detection of LOS sialylation was performed using western blot analysis with cholera toxin B-subunit (Sigma Aldrich, St. Louis, MO, USA) as previously described [34].

Cloning, Expression, and Purification of Recombinant FHA Protein in *E. coli*

The gene encoding the putative filamentous hemagglutination domain protein (FHA) was PCR-amplified from ST-4526 isolates H_0002 and H_0089 using primers (forward for H_0002: CAC.

CATGAAAAAGA TGAGTAAACATATAG, forward for H_0089: CACCATGGGAGTTTTGATT.

GGCAAAACAG, reverse: TAATTCTTCTTTAAAAC-TAGCGAAAT), directly cloned into the pBAD202 D-Topo vector, and transformed into *E. coli* LMG194 (Life Technologies).

Table 1. MLST scheme of *C. jejuni* isolates from different sources in Japan.

Source	No . isolate	Clonal Complex (CC)	%	STs*	%	Source	No. isolate	Clonal Complex (CC)	%	STs*	%
Human	100	CC-21	27.0	ST-50	9.0	Poultry	61	CC-21	16.4	**ST-4621**	6.6
				ST-8	3.0					ST-4526	4.9
				ST-21	4.0					ST-50	4.9
				ST-1360	1.0			CC-45	16.4	ST-3727	8.2
				ST-4526	7.0					ST-679	3.3
				ST-451	1.0					ST-45	3.3
				ST-4253	1.0					ST-845	1.6
				ST-4614	1.0			CC-48	11.5	ST-918	9.8
		CC-48	11.0	ST-48	7.0					ST-453	1.6
				ST-918	1.0			CC-443	8.2	ST-51	4.9
				ST-38	1.0					ST-440	1.6
				ST-453	1.0					**ST-4615**	1.6
				ST-918	1.0			CC-574	8.2	ST-305	8.2
		CC-42	6.0	ST-42	5.0			CC-353	6.6	**ST-4616**	3.3
				ST-447	1.0					ST-404	1.6
		CC-257	6.0	ST-257	4.0					ST-4052	1.6
				ST-361	2.0			CC-607	6.6	ST-4108	4.9
		CC-22	5.0	ST-22	4.0					ST-4600	1.6
				ST-1947	1.0			CC-354	4.9	ST-354	3.3
		CC-45	5.0	ST-45	4.0					**ST-4617**	1.6
				ST-3727	1.0			CC-460	3.3	ST-461	1.6
		CC-443	5.0	ST-51	2.0					ST-460	1.6
				ST-440	3.0			CC-22	3.3	ST-22	3.3
		CC-353	4.0	ST-4052	2.0			Others	14.8	**ST-4623**	6.6
				ST-353	1.0					ST-4389	3.3
				ST-2076	1.0					**ST-4618**	1.6
		CC-49	3.0	**ST-4624**	1.0					**ST-4620**	1.6
				ST-4613	2.0					**ST-4622**	1.6
		CC-354	3.0	**ST-4611**	1.0						
				ST-4623	1.0	**Cattle**	**51**	**CC-21**	**27.5**	**ST-21**	**7.8**
				ST-354	1.0					ST-806	15.7
		CC-45	2.0	ST-45	2.0					ST-50	3.9
		CC-460	2.0	ST-460	1.0			CC-42	19.6	ST-42	19.6
				ST-535	1.0			CC-22	9.8	ST-22	9.8
		CC-52	1.0	ST-52	1.0			CC-61	7.8	ST-61	7.8
		CC-61	1.0	ST-61	1.0			CC-48	7.8	ST-38	7.8
		CC-206	1.0	ST-46	1.0			CC-283	5.9	ST-4063	5.9
		CC-574	1.0	ST-305	1.0			CC-403	3.9	ST-933	3.9
		CC-658	1.0	ST-658	1.0			CC-257	2.0	ST-257	2.0
		Others	16.0	ST-922	6.0			Others	15.7	ST-58	15.7
				ST-2276	1.0						
				ST-4612	1.0						
				ST-407	4.0						
				ST-2328	2.0						
				ST-4390	2.0						

*Novel STs found in this study were shown in bold.
doi:10.1371/journal.pone.0048394.t001

Table 2. Summary of the ST-4526 isolates determined in this study.

Isolate	Source	Isolation date	Isolation area	Serotype		PFGE group	LOS sialylation	Antibiotic resistance
				Lior	Penner			
H_0002	Human	May, 2005	Tokyo	LIO4	O:2	a	–	NA, CPFX, LVFX
H_0055	Human	Jun., 2005	Osaka	LIO27	O:2	d	–	NA, CPFX, LVFX
H_0059	Human	Aug., 2005	Osaka	LIO27	O:2	d	–	NA, CPFX, LVFX
H_0060	Human	Oct., 2005	Osaka	LIO4	O:2	a	–	NA, CPFX, LVFX
H_0089	Human	Jul., 2006	Osaka	LIO4	O:2	b	–	NA, CPFX, LVFX
H_0092	Human	Sep., 2006	Osaka	LIO4	O:2	b	–	NA, CPFX, LVFX
H_0097	Human	Oct., 2006	Osaka	LIO4	O:2	a	–	NA, CPFX, LVFX
P_0016	Poultry	April, 2005	Yamaguchi	LIO4	O:2	c	–	NA, CPFX, LVFX
P_0017	Poultry	June, 2005	Yamaguchi	LIO4	O:2	c	–	NA, CPFX, LVFX
P_0053	Poultry	July, 2005	Aichi	LIO4	O:2	c	–	NA, CPFX, LVFX

PFGE groups were corresponded to those in Fig. 3.
doi:10.1371/journal.pone.0048394.t002

The expression of the His-tagged protein was induced in the presence of 0.1% L-arabinose according to the manufacturer's instructions. The rFHA protein was purified using Ni-NTA resin (QIAGEN) according to the manufacturer's instructions.

Binding Affinity of rFHA to Heparan Sulfate

A total of 5 µg of heparan sulfate from porcine intestine (Sigma Aldrich) in sodium carbonate buffer (pH 9.6) was captured onto a 48-well tissue culture plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland) at 4°C overnight. After washing with phosphate buffered saline (PBS, pH7.4) (Life technologies), 100 µg and serial dilutions of rFHA protein were added, and the plates were incubated at room temperature for 2 h. Following three washes with PBS, the bound proteins were solubilized in 20 mM Tris-HCl (pH 7.4) containing 0.1% SDS, and the protein concentrations were measured chronometrically using a Bradford assay.

Cell Adhesion/Invasion Assay

Caco-2 cell adhesive/invasive assays were performed as described [35]. Briefly, the cells were infected with *C. jejuni* at an multiplicity of infection (MOI) of 100, and incubated for 6 h. The wells were gently washed three times with sterile PBS (pH 7.4) (Life technologies), and the cells were detached using 0.2% saponin in PBS for 20 min. The suspensions were subsequently plated onto MH agar to count the colony-forming units (CFU). To enumerate the numbers of intracellular bacteria, a gentamicin protection assay was conducted after the same incubation period as previously described [35]. To minimize the effect of motility on cell adhesion, the culture plate was spun down (500×*g*, 3 min) immediately after the bacterial inoculation to synchronize the bacteria onto the cell surface. To examine the blocking effect of rFHA, the cells were pre-treated for 30min with 0, 5, or 20 µg of rFHA derived from the H_0002 isolate prior to *C. jejuni* infection.

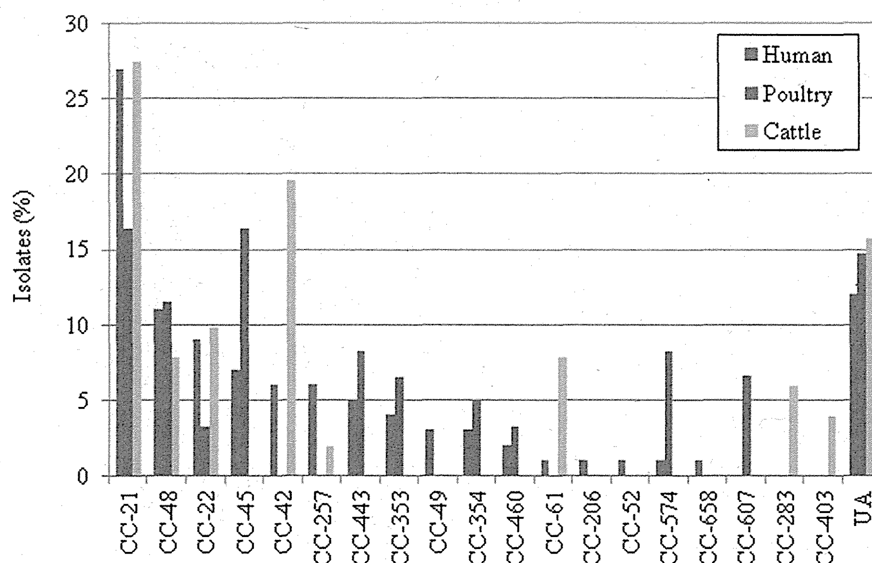
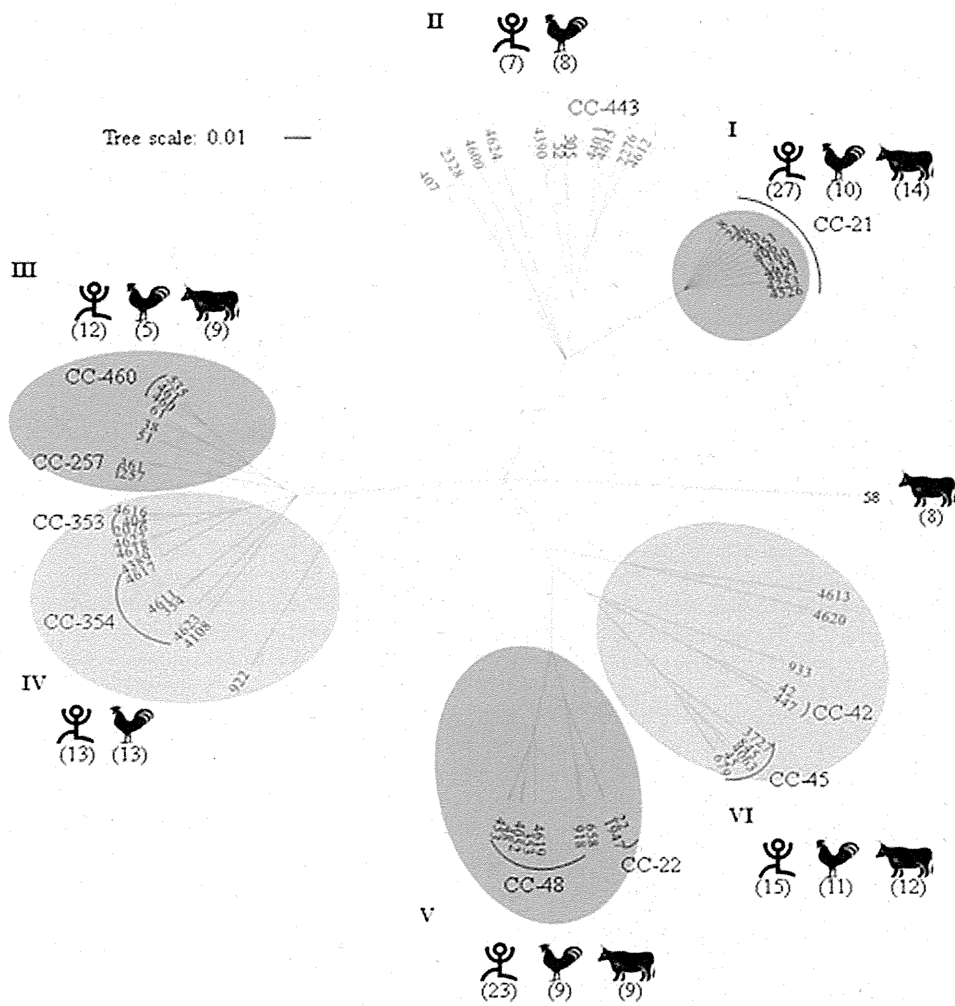


Figure 1. Frequency distribution of the *C. jejuni* clonal complexes (CCs) isolated from humans, poultry, and cattle in Japan (n = 212), which were classified into 62 sequence types (STs). UA represents isolates unassigned to any CCs.
doi:10.1371/journal.pone.0048394.g001

A



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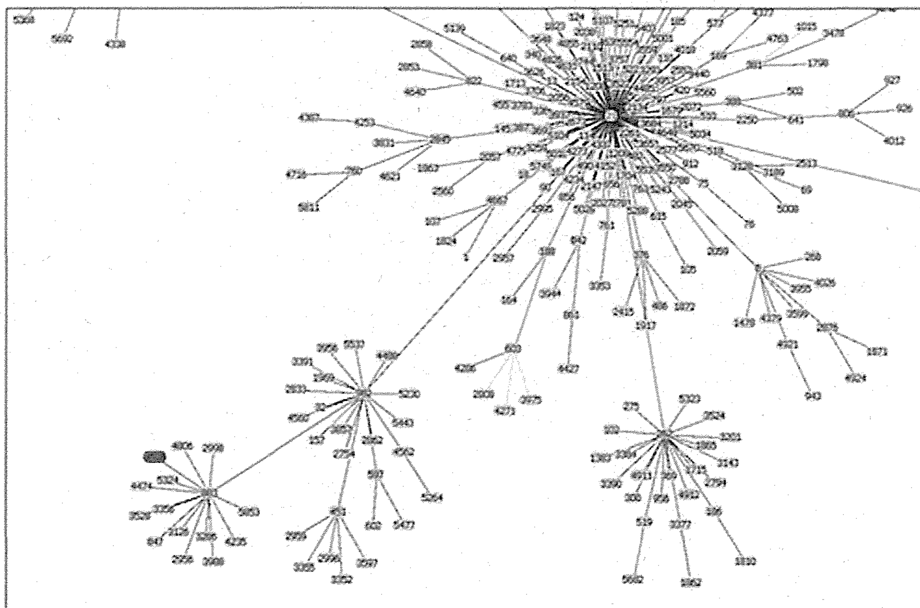


Figure 2. Genetic population and phylogeography of the *C. jejuni* MLST dataset. (A) The ClonalFrame analysis data for the 62 STs in the 212 *C. jejuni* isolates in Japan. The numbers of isolate sources per subgroup (cluster I to VI) are shown in the parenthesis, with illustrations (human, poultry, and cattle). (B) Enclosed graphic for the Global Optimal eBURST (goeBURST) analysis of *C. jejuni* CC-21 to assess the clonal lineage of ST-4526 (red) from the ancestor ST-21. The entire data is shown in supplemental Figure S1.
doi:10.1371/journal.pone.0048394.g002

The numbers of cell-associated bacteria were counted after gentle washing with PBS.

Antibiotic Susceptibility Test

Disk diffusion tests were conducted to determine the susceptibilities of the *C. jejuni* ST-4526 isolates to nalidixic acid (NA), ciprofloxacine (CPFX), levofloxacin (LVFX), erythromycin (EM), ampicillin (ABPC), gentamycin (GM), and chrolamphenicol (CM) according to the supplier's instructions (Becton Dickinson).

DNA Uptake and Natural Transformation Efficiency Assays

The DNA uptake assay was performed essentially as described [36]. Briefly, 0.1 μg of plasmid pRY108 DNA [37] labeled with [α-^{32}P]dCTP was co-incubated with approximately $4.5–5.8 \times 10^7$ CFU of *C. jejuni* MH-broth suspension for 30 min. After washing and DNase I treatment, the intrabacterial uptake of radioactivity was measured using a scintillation counter. The transformation efficiency was also assayed in parallel according to the method of Wang and Taylor [38]. Briefly, equal amounts of *C. jejuni* MH-broth suspension as prepared above were incubated with 1 μg of chromosomal DNA from the *C. jejuni peb4* mutant (carrying Cm-resistant cassette) [25] on MH agar supplemented with 5% horse blood for 6 h. The bacterial cells were subsequently resuspended in 1 ml of MH broth, and 50 μl of the bacterial suspension were spread onto MH agar plates supplemented with or without Cm (20 μg/ml). The numbers of colonies grown on each plate were comparatively measured to determine the transformation efficiency.

Statistics

The START ver.2 program [39] was used to determine the number of informative and parsimony informative sites, MLST alleles for each gene, and polymorphic sites according to the website guidelines (http://pubmlst.org/software/analysis/start2/). The genetic distances and rates of synonymous (*dS*) and non-synonymous substitutions (*dN*) were calculated using the MEGA ver.5 (Molecular Evolutionary Genetic Analysis) program [40]. The MEGA software was also used to draw amino acid alignment for the FHA protein. The data from all phenotypic assays represent the means ± standard deviations (SD) from at least three independent experiments.

Results

Summary of the *C. jejuni* MLST Dataset

A total of 212 *C. jejuni* isolates from human (n = 100) and animal sources (61 poultry and 51 cattle) collected in Japan from 2005–2006 were subjected to MLST analysis targeting seven genes (*aspA, glnA, gltA, glyA, pgm, tkt, uncA*). A total of 185 isolates (87.3%) were classified into 62 STs with 18 clonal complexes (CCs), and 11 STs (n = 27, 12.7%) were not assigned to any CCs (Tables 1 and S1); 42.5% of the strains belonged to three predominant CCs: CC-21 (n = 51, 24.1%), CC-48 (n = 22, 10.4%), and CC-45 (n = 17, 8.0%). The *pgm* gene yielded the greatest number of alleles, with 23 different alleles (Table S2), and *uncA* retained the greatest synonymous means (123.8) and lowest *dN/dS* value (0.0106). The total site variation within each gene ranged from 3.0% (*gltA*) to

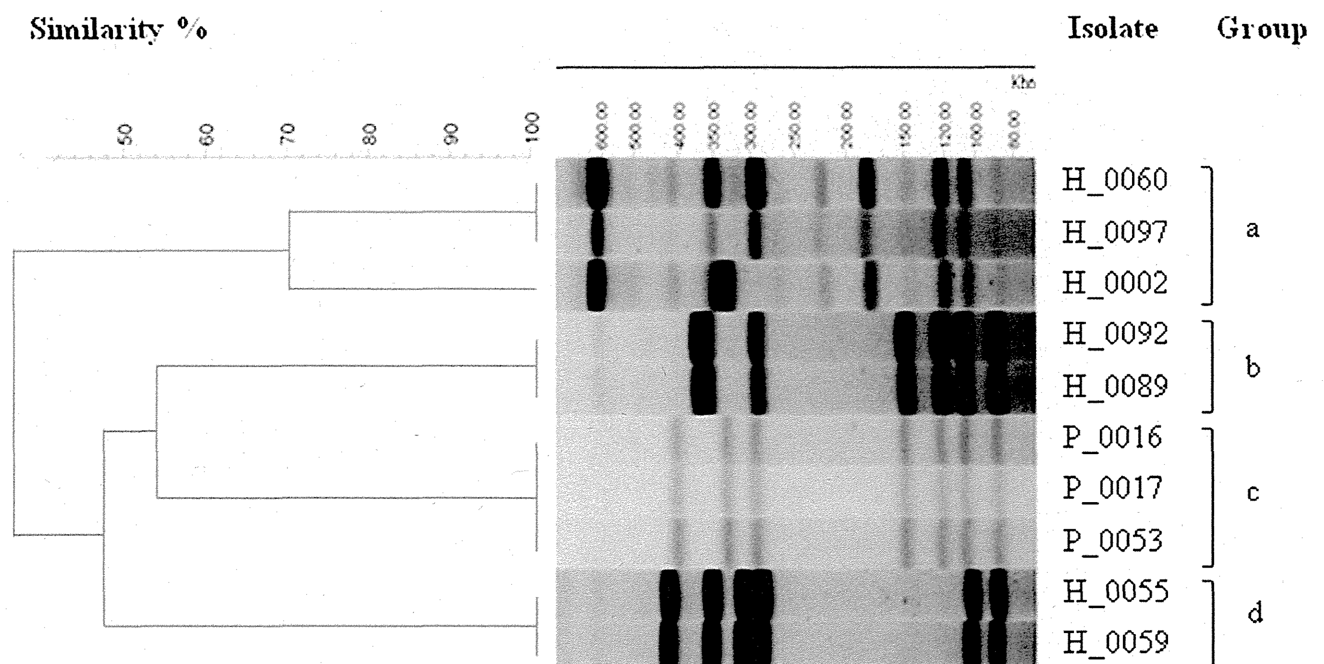


Figure 3. Pulsed-field gel electrophoresis (PFGE) analysis of *C. jejuni* ST-4526 isolates. The samples were *SmaI*-digested, followed by loading onto a 1% agarose gel. The isolates were divided into 4 groups, with a cut-off value of 70% similarity.
doi:10.1371/journal.pone.0048394.g003

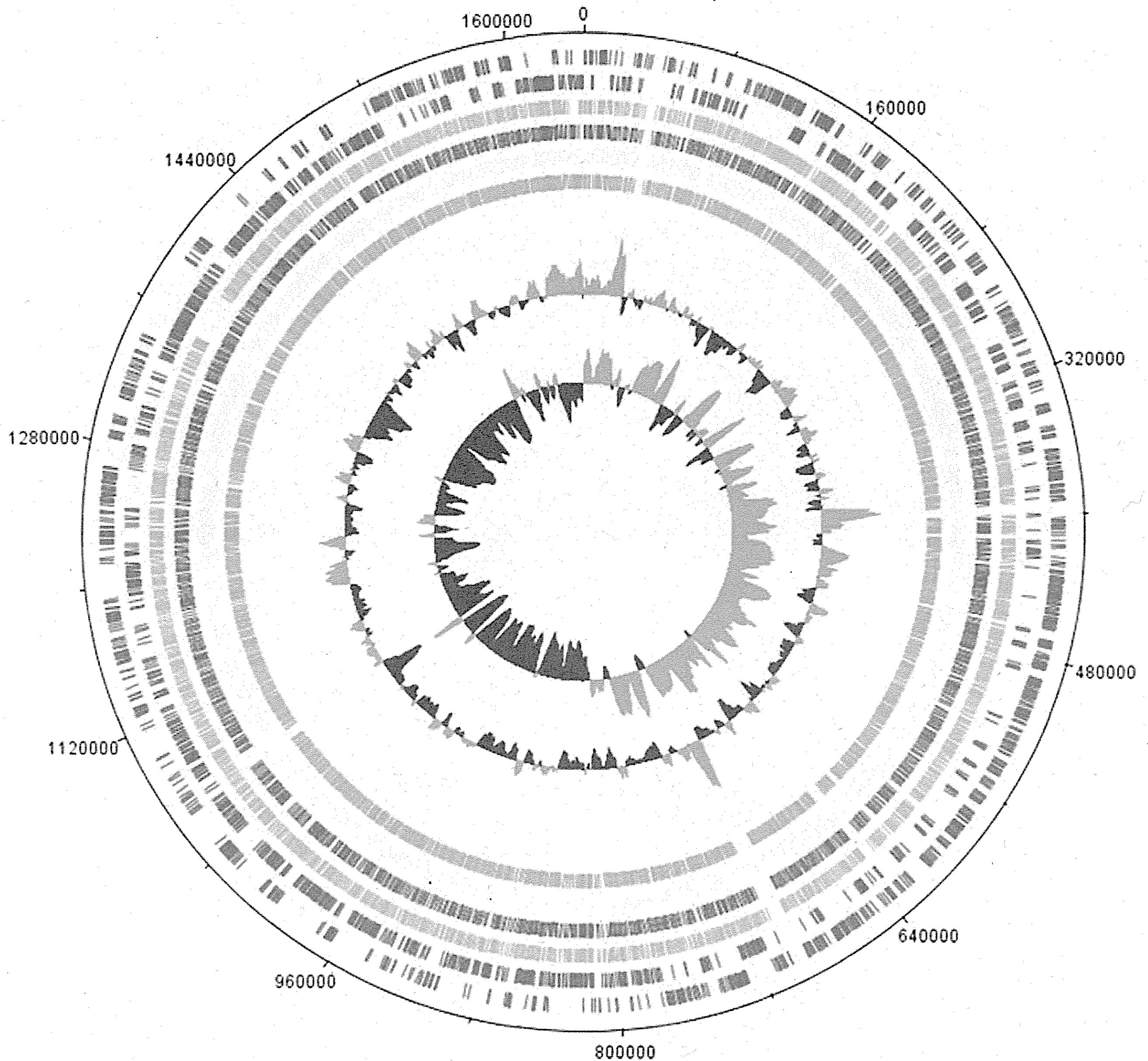


Figure 4. Comparative genome analysis of *C. jejuni* ST-4526. The CDSs of the reference genome of strain NCTC11168 (accession No. AL11168) are shown in the two outer rings (red, clockwise and counterclockwise). The subsequent rings depict RAST-annotated CDSs in the genomes of IA3902 (accession No.CP001876) (grey), H_0002 (green), H_0089 (yellow), and P_0053 (orange). The innermost ring depicts the GC content variation and GC skew from the mean (60%) of the reference genome.
doi:10.1371/journal.pone.0048394.g004

14.9% (*uncA*) (Table S2). The host-by-host characteristics are summarized below.

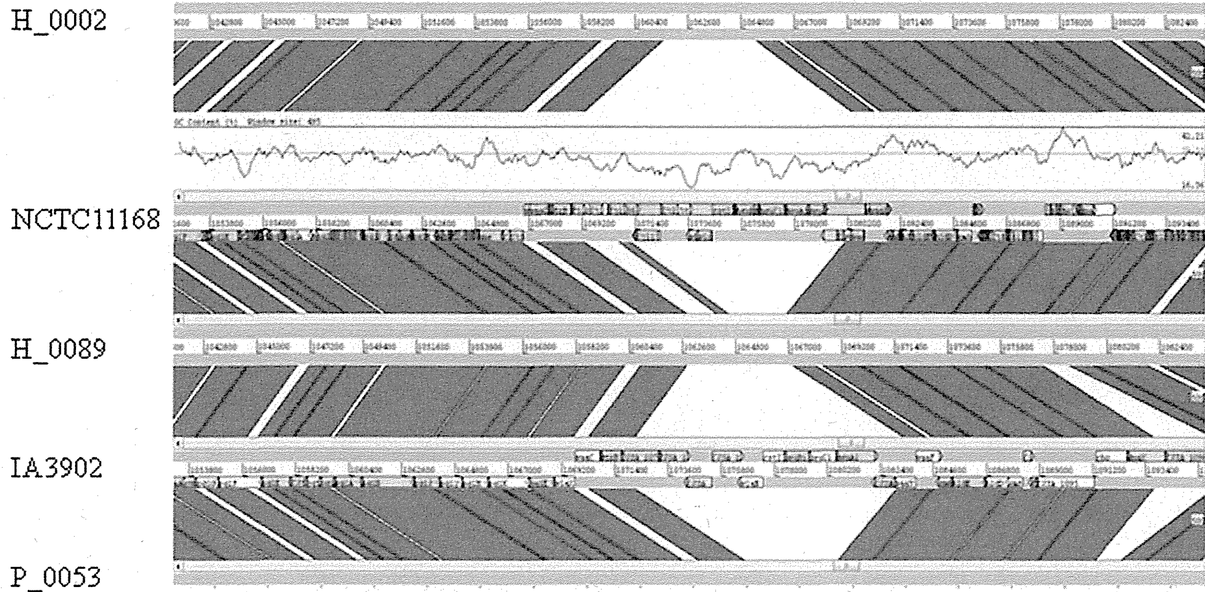
(i) Human clinical isolates

The human clinical isolates generated 43 STs (16 CCs plus 4 singletons), of which six STs were novel types (ST-4611 to 4614, 4623, and 4624) (Table 1, Fig. 1). Most of these novel types were represented by a single strain, except for ST-4613 (Table 1). A majority of the human isolates (81%) belonged to 10 CCs (CCs-21, −48, −22, −45, −42, −257, −443, −353, −49, and −354), of which 27% belonged to CC-21 (Fig. 1, Table 1). Within CC-21, the three most prevalent STs were ST-50 (n = 9, 33.3%), ST-22 (n = 8, 29.6%), and ST-4526 (n = 7, 25.9%) (Table 1).

(ii) Poultry isolates

The poultry isolates fell into 29 STs (10 CCs plus 6 singletons). CC-21 and CC-45 shared the highest (16.4%) coverage (Fig. 1, Table 1), in which ST-4526, one of the predominant STs among isolates from humans, was also represented by three poultry isolates (Table 1). A total of 9 novel STs were identified (ST-4615 to 4623), of which 4 isolates were associated with ST-4623 (Table 1). The most predominant STs were STs-918 (n = 6, 9.8%), −305 (n = 5, 8.2%), −4621 (n = 4, 6.6%), and −4623 (n = 4, 6.6%) (Table 1).

A



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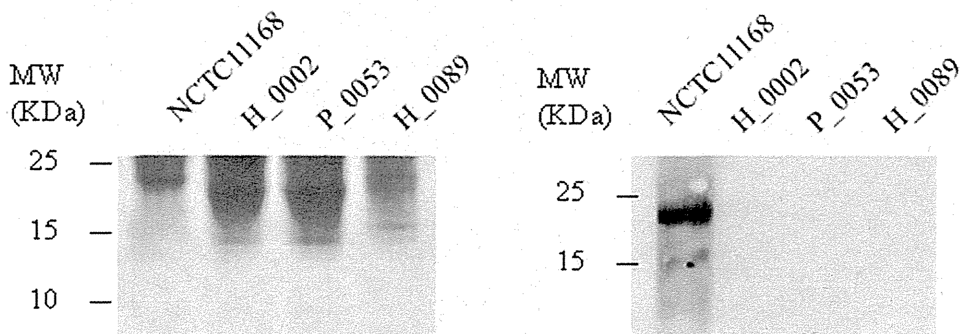

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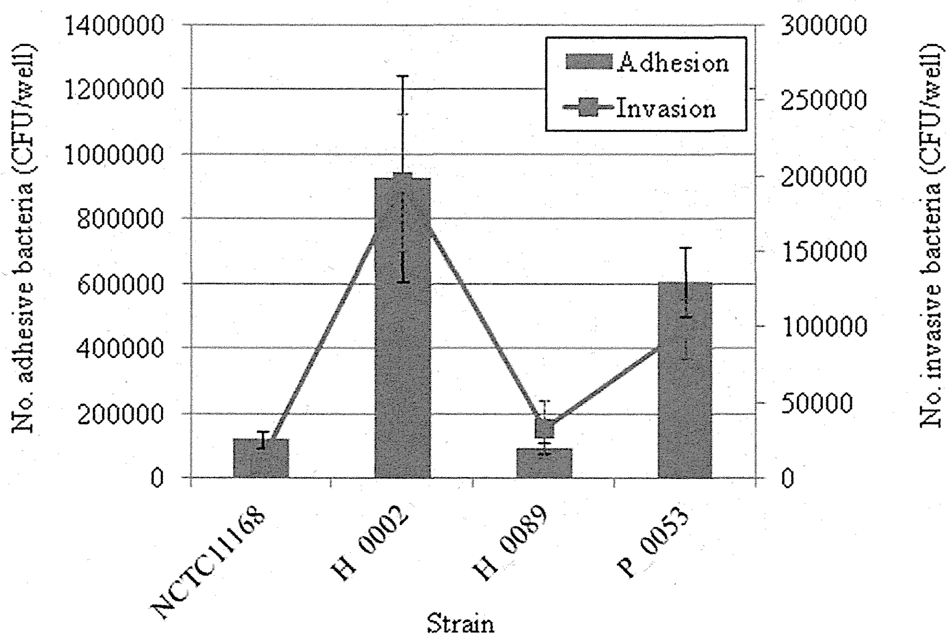

Figure 5. Genetic alteration in the lipooligosaccharide (LOS) modification locus in ST-4526. (A) Focused genetic alignment for the LOS locus among the NCTC11168, IA3902, H_0002, H_0089, and P_0053 strains, which was imaged using the Artemis Comparison Tool (ACT). (B) SDS-PAGE and detection of sialylation in *C. jejuni* LOS. The samples were extracted from NCTC11168, H_0002, H_0055, H_0089, and P_0053 strains and visualized using silver stain after loading onto 15% acrylamide gels (left panel). In parallel, the same samples on the acrylamide gels were subjected to western blot analysis using the GM1-reactive, HRP-conjugated cholera toxin B-subunit to detect sialylated LOS (right panel). (C) Cell adhesion and invasion properties of NCTC11168 and *C. jejuni* ST-4526 representatives. No adhesive or invasive bacteria were shown in either blue bar (means corresponding to the left Y-axis) or red lines (means corresponding to the right Y-axis), respectively.
doi:10.1371/journal.pone.0048394.g005

(iii) Cattle isolates

The 51 cattle isolates fell into 11 STs with 8 CCs (with one singleton, ST-58). Similar to the human and poultry populations, CC-21 shared the highest coverage (27.5%), and accordingly, CC-61 and CC-42 covered 19.6% and 7.8%, respectively (Fig. 1, Table 1). ST-42 and ST-806 shared either 19.6% or 15.7% coverage, respectively (Table 1). ST-58 was identified in 7 cattle isolates (Table 1), the source of which was commonly found in the Oita prefecture (Table S1).

Population Structure of the MLST Dataset

To reveal the population structure of the *C. jejuni* isolates, a ClonalFrame analysis was conducted. This phylogenetic program grouped the 62 STs into 6 clusters (I to VI) (Fig. 2A); one of the predominant CCs, CC-21, was grouped into Cluster I. No cattle isolates were included in Clusters II and IV, which frequently included CC-460, CC-257, CC-353, and CC-354 from human and poultry populations. ST-58 was only identified in the cattle population (n = 8) and showed a distinct lineage from the other STs. Clusters III, V, and VI were generated in three (human, poultry, and cattle) populations. Thus, these data revealed a partial host association of distinct CCs/STs.

Evolution Linkage of ST-4526 within CC-21

The MLST analysis revealed that ST-4526 was represented in 7 human and 3 poultry isolates, although their isolation areas and dates were diverse (Tables 1 and 2). Therefore, a search for this ST in the *Campylobacter* MLST database led to the identification of a single poultry isolate that was also isolated in Japan (Isolate ID 10249). For a more comprehensive analysis of the possible patterns of evolutionary descent, the goeBURST (global optimal eBURST) analysis [28] was carried out using a total of 547 STs belonging to CC-21 deposited on the *Campylobacter* pubMLST database (as of 2, August, 2012). This approach resulted that the ST-4526 showed one of the most distant lineage from the original (founding) ST-21 genotype, together with the genotypes originated only from human and poultry (ST-883 and the derivatives, Fig. 2B). Other 13 STs reported only from Japan (i.e. ST-4253, ST-4387, ST-5233) were classified into different clusters (Fig. S2). Together, these data support the idea that ST-4526 might be distinctly evolved from the ancestor.

Summary of Genomic Features of *C. jejuni* ST-4526 Strains

Given the potent thriving and unique evolutional lineage of ST-4526, all ST-4526 isolates from human and poultry (n = 10) were macrogenotyped using pulsed-field gel electrophoresis (PFGE) analysis to determine their genomic similarities. As shown in Fig. 3, these isolates were divided into 4 groups (a–d), in which 3 poultry isolates (P_0016, P_0017, P_0053; group c) were closely related to the human isolates H_0089 and H_0092 that belonged to group b. To further clarify the genomic similarities and characteristics that might account for the thriving of these bacteria, three representative isolates (H_0002, group a; H_0089, group b; P_0053, group c) were genome sequenced, resulting in contigs comprising 1,625,252 bp (H_0002), 1,627,144 bp (H_0089), and 1,626,331 bp (P_0053). The sequences were subsequently processed with the RAST annotation pipeline [30], which annotated 1,836 to 1,865 features (Tables S3–S5). The three ST-4526 genomes were compared with the genomes of NCTC11168 (ST-43/CC-21, accession No. AL11168) and IA3902 (ST-8/CC-21, accession No. CP001876). DNA Plotter depicted the comparative genome circular maps for the genomes discussed above (Fig. 4).

ST-4526 Expresses Asialylated forms of Lipooligosaccharides (LOS)

The genome alignments using ACT (Artemis Comparison Tool) combined with a Bi-BLAST comparison of *C. jejuni* ST-4526 strains with strains NCTC11168/IA3902 clearly showed significant overall synteny between the five genomes. However, the LOS modification locus was not conserved (Cj1137c to Cj1144c) (Fig. 5A, Table S6). In contrast to strain NCTC11168, the LOS of the 3 ST-4526 isolates did not react with the cholera toxin, a ligand for GM1-oligosaccharide structures (Fig. 5B). Sialylated LOS structure is known to contribute to cell invasion [41] and gastrointestinal colonization in poultry [42]. The Caco-2 cell adhesion/invasion assays indicated, however, that the H_0002 and P_0053 isolates exhibited greater cell adhesion and invasion properties than the H_0089 and NCTC11168 isolates (Fig. 5C), providing an idea that the LOS differences did not essentially contribute to cell adhesion/invasion, hence certain determinant(s) might preferentially promote cell adhesion and invasion in H_0002 and P_0053.

ST-4526 Expresses a Putative Adhesin Protein with a Filamentous Hemagglutination Domain (FHA)

The Bi-BLAST analyses further revealed that the region from Cj0738 to Cj0752 was highly diverse between ST-4526 and NCTC11168 (Table S6). The Island Viewer [32] subsequently predicted that the three ST-4526 isolates contained a putative island comprising the region from Cj0737 to Cj0744, which harbored genes which differed between ST-4526 and NCTC11168/IA3902 (Fig. 6A). The amino acid alignment analyses showed that the Cj0742-homologue filamentous hemagglutination domain protein (FHA) was expressed in isolates H_0002, P_0053 and strain CF93-3 (accession No. CJJCF936_0827), which encoded a 693 aa protein with 100% sequence similarity; notably, this protein was truncated in H_0089 and NCTC11168/IA3902, with 585 aa (lacking N-terminus) and 358 aa (lacking C-terminus), respectively (Fig. S3). Other strains also exhibited distinct aa sequences, ranging from 358aa (strains 84-25 and DFVF1099) to 633aa (strain 1213) (Fig. S3).

FHA functions as an adhesin in *Bordetella pertussis*, showing binding affinity to carbohydrates, such as heparan sulfate, distributed on epithelial cell surfaces [43]; therefore, we produced recombinant FHA (rFHA) protein in *E. coli* (Fig. S4) and measured its affinity to heparan sulfate. The results showed that the rFHA from the H_0002 isolate showed greater binding