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RESEARCH ARTICLE

Genetic Similarity Using MLST Amongst *Campylobacter jejuni* Isolates from Children with Diarrhea Symptoms and Broilers

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Abstract The aim of the present study was to investigate the genetic similarity amongst *Campylobacter jejuni* isolates from children with diarrhea symptoms and broilers in Chiang Mai, Thailand during 2002–2007. Sequence types of 12 isolates from children with symptoms of diarrhea and 9 isolates from broilers and other 42 Thai sequence types were determined by multilocus sequence typing. Cluster analysis was performed to determine the molecular relatedness of the isolates. Results showed diverse sequence types of *C. jejuni* isolated from both broilers and children. One isolate each from a broiler and a child shared a single sequence type (574). The majority of 13/16 sequence types belonged to previously reported sequence types in Thailand and two previously reported human sequence types were identified in broilers. The authors conclude that there is evidence of a genetic relationship between *C. jejuni* isolated from humans and broilers. MLST analysis of *C. jejuni* suggested that broilers are one of the sources of human infection in Chiang Mai, Thailand.

Keywords *Campylobacter jejuni* · Chicken · Children · Multilocus sequence typing

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Introduction

Food poisoning is an important health issue in several countries including Thailand. Studies in Thailand have shown that acute diarrhea in children <12 years old is commonly caused by *Campylobacter* sp. (28 %), *Salmonella* sp. (18 %), *Shigella* sp. (9 %) and *E. coli* (6 %) [1]. A study conducted in Chiang Mai, the northern region of Thailand, revealed that *C. jejuni* was most commonly found in patients [2]. This species can be transmitted from infected poultry farms along the processing chain to consumers [3]. The farm environment is known to be a potential source of the occurrence of this bacterium in food animals [4]. In northern Europe, *C. jejuni* has been found in the environment, water resources and livestock keeping areas [5]. Since there have been a variety of sources of infection and the outbreaks usually consisted of sporadic cases, the sources and transmission routes of outbreaks are difficult to trace [6]. In order to determine the source of infection, several molecular typing schemes have been developed for characterizing and tracing *Campylobacter* spp.

The multilocus sequence typing (MLST) technique for molecular fingerprinting of the *Campylobacter* genome has one important advantage as compared with other molecular techniques that global MLST databases can be used for identifying the potential source of infection in outbreak investigations [7–11]. MLST is also considered to have a higher discriminatory index than other molecular methods [12].

In 2015, the *Campylobacter* Multi Locus Sequence Typing website (http://pubmlst.org/campylobacter/) provided access to 7797 profiles of *C. jejuni* isolated globally from humans [13]. The aim of the present study was to investigate the genetic relationship between *C. jejuni*

isolated from animals and humans in Chiang Mai, Thailand by comparing sequence types (STs) of isolates from the same geographic area and period during 2002–2007.

Material and Methods

Nine *C. jejuni* isolates from broilers and 12 from children with diarrhea symptoms from the Chiang Mai region, Thailand during the period 2002–2007 were included in the study. Briefly, broiler samples were collected at the slaughterhouse and again at the markets while stool samples from children with diarrhea symptoms were asked by directly contacting for participation while the child was hospitalized [2]. All sampling sites were located in the Chiang Mai of northern Thailand. Isolation and identification of these *Campylobacter* bacteria was performed by selective culture in microaerophilic atmosphere and polymerase chain reaction [2]. All isolates were kept at −70 °C in cryovial tube. Prior to DNA extraction, cultures were removed from −70 °C and allowed to thaw at room temperature. These were then spread out onto a Brucella agar plate (Oxoid®, UK) to produce discrete colonies and incubated at 37 °C for 72 h under microaerobic conditions with a CO_2 generator (Anaerocult® C; Merck, Germany) (5). DNA extraction was performed using the cetyltrimethyl ammonium bromide (CTAB) precipitation method, as described previously [14].

MLST was performed using standard primers as described in Table 1 using selective amplification of the following seven genes and determining their sequence: aspartase A (*asp*A), glutamine synthetase (*gln*A), citrate synthase (*glt*A), serine hydroxymethyl transferase (*gly*A), phosphoglucomutase (*pgm*A), transketolase (*tkt*A), ATP synthase subunit (*unc*A) [9, 10, 15]. PCR reactions were carried out in a final volume of 50 µl, each mixture including: 4 µg of *Campylobacter* DNA, 0.2 µM of forward and reverse primers, 1X PCR buffer, 2 mM MgSO4, 0.2 mM deoxynucleoside triphosphates, and 1 U of High Fidelity Platinum *Taq* DNA polymerase (Invitrogen). Fluorescent dye-determinator sequencing was performed using the ABI Prism 3730 XL DNA sequencer method (Bio Basic Inc, Canada). The sequences were submitted to the international databases on the Campylobacter Multi Locus Sequence Typing website (http://pubmlst.org/campylobacter). All allelic profiles were recorded and a sequence type (ST) and clonal complexes (CC) were assigned [16]. In addition, other 42 sequence types of Thai isolates were included for analysis.

Comparison of allelic profiles and ST was done using the tools available online at MLST public database (http://pubmlst.org/perl/mlstanalyse/mlstanalyse.pl?site=pubmlst). Phylogenetic trees were generated from allelic profile data based on the neighbor-joining and UPGMA method using the tree drawing tool of the Bionumerics® version 4.0 software (Applied Maths BVBA, Belgium). Trees were displayed using the Phylodendron software (http://iubio.bio.indiana.edu/soft/molbio/java/apps/trees/). STs found in this study and STs of 42 additional isolates of *C. jejuni* available on the public MLST databases were grouped together using the 'based upon related sequence types' (BURST) algorithm version 1.00 (http://pubmlst.org/analysis/burst/burst.shtml) with a stringent group definition (4/7 shared alleles). Isolates in the groups defined by BURST were considered to belong to a single clonal complex [13]. BURST uses a model of bacterial evolution in which an ancestral (or founding) genotype increases in frequency in the population, and diversifies to produce a cluster of closely related genotypes that all descend from the founding genotype. The BURST algorithm first identifies mutually exclusive groups of related genotypes in the population such as MLST, and attempts to identify the founding genotype of each group. The algorithm then predicts the descent from the predicted founding genotype to the other genotypes in the group. ST with at least two assigned descendent single locus variants (SLV) is defined as a subgroup founder [13].

Table 1 Primers

Locus	Primers		Amplicons
	Forward	Reverse	
asp	5′-AGTACTAATGATGCTTATCC-3′	5′-ATTTCATCAATTTGTTCTTTGC-3′	899
gln	5′-TAGGAACTTGGCATCATATTACC-3′	5′-TTGGACGAGCTTCTACTGGC-3′	1262
glt	5′-GGGCTTGACTTCTACAGCTACTTG-3′	5′-CCAAATAAAGTTGTCTTGGACGG-3′	1012
gly	5′-GAGTTAGAGCGTCAATGTGAAGG-3′	5′-AAACCTCTGGCAGTAAGGGC-3′	816
pgm	5′-TACTAATAATATCTTAGTAGG-3′	5′-CACAACATTTTTCATTTCTTTTTC-3′	1150
tkt	5′-GCAAACTCAGGACACCCAGG-3′	5′-AAAGCATTGTTAATGGCTGC-3′	1102
unc	5′-ATGGACTTAAGAATATTATGG C-3′	5′-GCTAAGCGGAGAATAAGGTGG-3′	1120

Results and Discussion

Amongst 21 *C. jejuni* isolates from both chicken and children from Chiang Mai, Thailand during 2002–2007, 16 STs were identified in the present study (Table 2). Of these, 13 STs (80 %) were previously found in Thailand. ST-574 was found both in human and in chicken isolate. ST-2276 and ST-2921 previously found only in humans in Thailand were also identified from chickens (Table 3). ST-2276 seemed to be indigenous to broilers in Thailand as reported in previous studies [17, 18]. ST-1232 and ST-1919 previously isolated from humans elsewhere were found in the present study in chickens.

Eleven STs can be grouped into 8 CCs including 13 *C. jejuni* isolates. One out of 8 CCs (12.5 %) included isolates from both chickens and children with diarrhea while 3 out of 8 CCs (37.5 %) included only isolates from chickens. Five unassigned STs included one from chicken and the others from humans. Additionally, possible routes of transmission of *C. jejuni* among chicken and human isolates have been determined by Levesque et al. [6]. The study suggested that sporadic *C. jejuni* infections in humans are frequent due to the sources other than chickens.

The phylogenetic tree (Fig. 1) showed that *C. jejuni* isolates from chickens and children with diarrhea were clustered together. BURST grouping also showed that 13 out of 21 isolates can be grouped into 3 groups with similarity >40 % in groups 1 and 3, and in group 2 more than 70 %. The majority of isolates that did not belong to any ancestral group were isolated from diarrhea children (6/8).

A total of 63 isolates, 21 isolates from Chiang Mai and 42 from Thai isolates, were analyzed (Table 4). MLST sequences were grouped into 55 STs and 3 groups by BURST. Group 1 consisted of 39 STs from 45 isolates and ST-464 was the central ST representing 6 subgroups. Groups isolated from human samples only were ST-464, ST-2284 and ST-2922. In case of ST-2922, which had ST-2274, this ST could be found in chickens from Belgium and Thailand also [17, 18], but ST-2284 was found in Thai people only. Group 1 and 3 results (Table 4) showed that all samples were isolated from humans including those 8 STs which could not be joined with any of these groups. Group 2 samples were isolates from humans in Thailand only.

The results showed genetically diverse STs of *C. jejuni* isolated from both chickens and children from northern Thailand. Amongst 21 samples with 16 different STs from the northern region of Thailand, ST-2921, ST-2276 and ST-2332 were reported from Thailand [9, 19] in the present study. According to the BURST and CC results, the ST-52 and ST-607 complexes were the most common *Campylobacter* species in Thailand. In New South Wales, Australia, the most common CCs were ST-48, ST-257, ST-345 and ST-21 complexes [20]. The ST-21 complex rarely found in Thailand, included 2 *Campylobacter* isolates from children in the northern region. The most commonly found

Table 2 Genetic character of *C. jejuni* using MLST

Clonal complexes	ST	Chicken	Diarrhea children
ST-21 complex	50		MC-50, MC-51
ST-22 complex	22		MC-43, MC-74
ST-52 complex	1919	Chick-562	
	4357*		NP-33
	4363*	Chick-380	
ST-353 complex	1232	Chick-553	
ST-354 complex	354		MC-79
	4358*	Chick-52	
ST-443 complex	51		NP-2
ST-574 complex	574	Chick-206	NP-30
ST-607 complex	2921	Chick-542, Chick-610	
Unassigned	436		NP-12
	464		MC-55
	1726		MC-30
	2276	Chick-568, Chick-572	
	2332		MC-48

Bold newly found sequence type

* Newly found allele of *gly*A gene

Table 3 Comparison of the samples' number in each STs found in the present study and the ST percentage from other sources in the database (February 2012; http://pubmlst.org/campylobacter/)

ST	Number found in human in this study	Number found in chicken in this study	Percentage of isolates from each source							No. found in Thailand	No. found in database
			Human	Chicken	Chicken meat or visceral organs	Cows	Wild birds	Environment	Others		
22	2		82.26	1.61	1.61	4.84	–	3.23	4.84	2	62
50	2		70.75	2.83	19.81	–	–	–	6.60	2	106
1919		1	50.00	50.00	–	–	–	–	–	1	2
354	1		86.21	3.45	3.45	–	–	–	6.90	1	29
51	1		97.87	2.13	–	–	–	–	–	1	47
574	1	1	46.15	50.77	–	–	1.54	–	1.54	2	66
2921		2	33.33	66.67	–	–	–	–	–	3	3*
436	1		71.43	–	–	–	28.57	–	–	1	7
464	1		90.91	–	9.09	–	–	–	–	1	11
2276		2	33.33	66.67	–	–	–	–	–	3	3*
1232		1	50.00	50.00	–	–	–	–	–	1	2
1726	1		100.00	–	–	–	–	–	–	1	2
2332	1		100.00	–	–	–	–	–	–	2	2*
4357	1		100.00	–	–	–	–	–	–	1	1*
4358		1	–	100.00	–	–	–	–	–	1	1*
4363		1	–	100.00	–	–	–	–	–	1	1*

Bold newly found sequence type in this study

* The data which appeared to be found in Thailand only

STs in avian species in Europe, USA, Canada and Australia were ST-21 and ST-45 [9], which could not be detected in broilers in Thailand [17, 18]. A study conducted in Australia from January 1991 to July 1993 reported that amongst a group of ST-607 complex isolates from human campylobacteriosis patients some individuals had a travelling history to Thailand where this was one of the most commonly occurring complex [20].

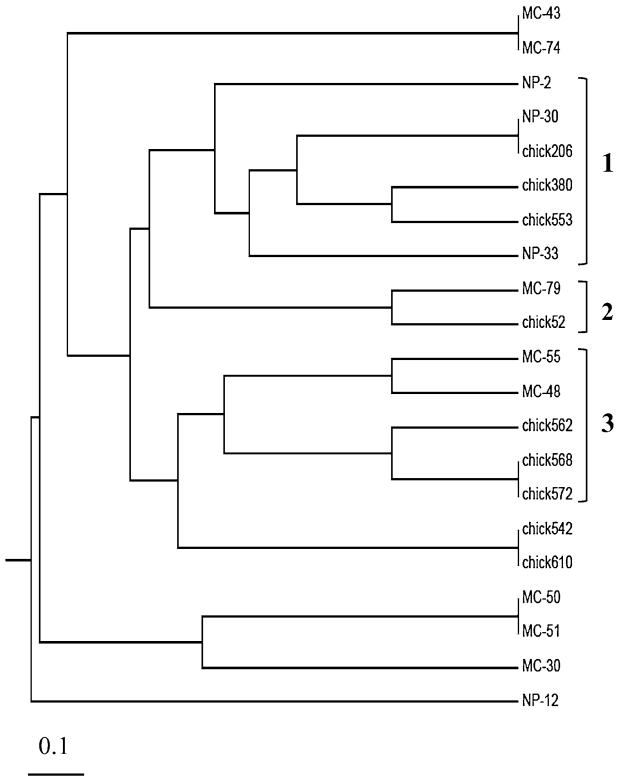

Fig. 1 UPGMA Phenogram of *C. jejuni* from the northern region of Thailand

According to BURST analysis results, most of the chicken isolates belonged to subgroup ST-2940. Interestingly, the ST-2921 belonged to ST-2940 subgroup among human samples. The ST-2276 and ST-2921 previously found in humans in Thailand were also found in broiler samples in the present study. Furthermore, ST-4035 which belonged to the ST-2941 subgroup was isolated from a Guillain–Barre syndrome patient. The finding of ST-436 both in human and wild starling bird samples indicated that wild birds can be a source of infection (Table 2). Both data from chicken samples in the present study and those from the database indicated that there might be a source of infection in humans from sources other than broilers and chicken products. Gormley et al. [21] found the genetic character of *C. jejuni* in human which was different from those in broilers. This situation could be because of poor hygiene behavior of consumers. The infections have occurred during the cooking process or during meals from several contamination sources including wild bird feces, and this could explain the increase genetic changes and genetic variation in some human beings [22].

The similarity between *C. jejuni* found in broilers and humans suggests the occurrence of transmission between broilers and humans. This indicates that both biosecurity on broiler farms and personal hygiene amongst humans needs to be strengthened. Although it would be rare for the children under 1 year of age to come into direct contact with chicken, there may be indirect transmission, for example by nurses, food with chicken ingredients or by *C. jejuni* contaminated water or sand [6]. *C. jejuni* has also been found in the intestine of domestic animals [19]. Appropriate levels of hygiene in child nurseries are essential, it is being neglected since infection in adults usually occurs asymptomatically. *C. jejuni* infection in infants can result in severe clinical signs including bloody

Table 4 Burst grouping result of all *C. jejuni* MLST found in Thailand (n = 63)

BURST Group	Number of STs	Number of isolates	Source of ancestral ST (human/animal)	Number of isolates from human	Number of isolates from animal
Group 1*	39	45	ST-464 (human)	36	9
Subgroup					
ST-354	3	3	2	1	
ST-2940	3	5	3	2	
ST-2941	11	12	9	3	
ST-464	5	6	6	0	
ST-2284	4	4	4	0	
ST-2922	3	3	3	0	
Group 2	5	5	–	5	0
Group 3	3	4	–	4	0
Unassigned	8	9	–	9	0

* 10 STs in Group 1 could not be classified into any subgroup

diarrhea and intussusception or necrotizing enterocolitis. About 92 % of the under 1-year-old children with campylobacteriosis had bloody diarrhea and at least 5 outbreaks in the nurseries have been reported. The transmission came from nosocomial infection particularly from the use of medical equipment such as thermometers or bathtubs without an appropriate sanitization [23].

The genetic characteristics of *C. jejuni* isolates in Thailand showed a weak clonal structure. Further studies are needed to discover more chicken *C. jejuni* isolates, which are the main source of *C. jejuni*. It can relate all data grouped in CCs with the ungrouped data [19]. Collection of samples from environmental sources e.g. water resources, soil, sand, wild birds and domestic animals [6] would be helpful for determining other sources of *C. jejuni*. Furthermore, studies about virulence factors of *C. jejuni* should be conducted to improve the understanding of the virulence and pathogenesis of the *C. jejuni* in human beings. Although MLST is a relatively expensive method, it has the advantage that it can be performed easily if given availability of genetic material and appropriate PCR equipment [12]. The utility of MLST for molecular epidemiological analysis depends on the availability of the number of gene profiles that can be used for comparative purposes, and it is therefore essential to submit any sample to internationally accessible databases.

Conclusion

There was an evidence of genetic relationship between *C. jejuni* isolated from human beings and broiler. MLST analyses of *C. jejuni* suggested that broiler might be one of the source of human infection in Chiang Mai, Thailand.

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Compliance with Ethical Standards

Conflict of interest All the authors declare that they have no conflict of interest in the aspect of either financial or personal relationships with other people or organizations that could inappropriately influence their work.

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