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A Literature Survey of *Campylobacter* Contamination in Retail Poultry Meats and By-Products in the World

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Keywords: *Campylobacter*, *C. coli*, *C. jejuni*, Literature survey, Retail poultry meats and by-products

Introduction

Campylobacter species are common bacterial Pathog-ene associated with human gastroenteritis worldwide (8). Contaminated raw or undercooked poultry meats and/or by-products are particularly important to cause food-borne campylobacteriosis in humans (2). There are many reports describing *Campylobacter* Contamination in retail poultry meats and/or by-products in the world. For comparing the contamination levels among the areas or countries, we performed the literature survey of *Campylobacter* contamination in retail poultry meats and by-products worldwide.

Materials and Methods

We searched for the papers which described the retail poultry meats and by-products contaminated with *Campylobacter* spp. by using the combination of two sets of keywords, which were "*Campylobacter*" and "poultry or chicken" in the following databases: JSTPlus, JMedPlus, PubMed, ScienceDirect, and Japana Centra Revuo Medicina. These databases were searched from July to August, 2007. The papers, which were published from 2002 to the time of the searches, describing *Campylobacter* contamination in retail poultry, but not in poultry at farms or at processing plants, were collected. Due to the insufficient space, the entire list of the collected papers can not be included in this manuscript.

Results and Discussion

We classified into 5 categories; poultry meats, poultry by-products, frozen poultry meats, frozen poultry by-products, and ground poultry meats. The average prevalence was calculated on the basis of either country or area or total.

In poultry meats, most of the countries showed 50% or more in prevalence of *Campylobacter* contamination (Table 1). Estonia and Belgium showed exceedingly lower prevalence, although the methods employed were common methods. Compared among the areas, Middle and South America and Oceania showed higher prevalence of contamination, although the number of papers was somewhat limited. On the other hand, former Soviet Union & Eastern Europe showed lower prevalence, mainly because exceedingly low prevalence was shown in Estonia.

Fewer investigations were reported concerning poultry by-products, frozen poultry meats, frozen poultry by-products, and ground poultry meats. Due to less information, these could not be compared on the country- or area-basis. On the average, the prevalence of poultry by-products was relatively higher than that of poultry meats. Frozen poultry meats and by-products showed lower prevalence compared with fresh poultry meats and by-products, respectively. This can be attributed to the report explaining that frozen conditions damage *Campylobacter* cells and decrease their viability (1, 6). The prevalence of *Campylobacter* contamination in ground poultry meats was lower than that in poultry meats. *Campylobacter* spp. are microaerobic bacteria and are damaged in the air (4). Therefore, it is speculated that *Campylobacter* cells contaminated might be exposed to the air and dead during and after the grinding process.

The frequency of *Campylobacter* spp. distribution among the isolates from retail poultry in various countries is listed in Table 2. In most of the countries, *C. jejuni* was the dominant species isolated from poultry, although the ratio of *C. coli* to *C. jejuni* was considerably different among the countries. Especially in Thailand and South Africa, *C. coli* was more frequently isolated from retail poultry meats and by-products compared to *C. jejuni*. *C. coli* was highly colonized even in poultry at farm level in Thailand (5, 7), *C. jejuni*, on the contrary, was the dominant species colonized in most of the countries (3), but the reasons are not certain.

We surveyed *Campylobacter* contamination in retail poultry meats and by-products in the world. In most of the countries, both industrialized and developing countries, a majority of retail poultry meats and by-products were contaminated with *Campylobacter* spp. *C. jejuni* was usually the dominant *Campylobacter* species isolated, but the ratio of *C. coli* to *C. jejuni* varied among the countries. Especially in Thailand and South Africa, *C. coli* was the dominant *Compylo-bacter* species isolated from retail poultry.

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Table 1 Prevalence of *Campylobacter* spp. in retail poultry in various countries

Samples	Countries	No. of references	No. of samples	Prevalence
poultry meats	**Total average**	**74**	**18817**	**57.7%**
	North America	**10**	**2472**	**62.8%**
	USA	7	995	70.4%
	Canada	3	1477	57.7%
	Middle and South America	**4**	**832**	**82.3%**
	Trinidad and Tobago	2	741	84.6%
	Argentina	1	14	92.9%
	Barbados	1	77	58.4%
	Europe	**43**	**10849**	**52.3%**
	Western Europe	**38**	**9929**	**55.4%**
	UK	14	4280	71.2%
	Germany	8	2462	45.6%
	Denmark	3	154	64.9%
	Austria	2	323	51.4%
	Ireland	2	904	51.1%
	Italy	2	185	80.0%
	Spain	2	252	59.5%
	Belgium	1	917	17.0%
	France	1	68	58.8%
	Sweden	1	45	42.2%
	Switzerland & Liechtenstein	1	339	25.1%
	Former Soviet Union & Eastern Europe	**5**	**920**	**19.2%**
	Estonia	2	739	8.1%
	Belarus & Russia	1	10	50.0%
	Bulgaria	1	135	76.3%
	Czech	1	1	100.0%
	Africa	**2**	**316**	**73.1%**
	Senegal	1	250	82.0%
	South Africa	1	66	39.4%
	Asia	**41**	**4088**	**60.6%**
	Thailand	4	186	73.1%
	Korea	3	1458	66.6%
	Vietnam	2	160	30.0%
	Japan	29	1544	58.6%
	Iran	1	121	62.8%
	Turkey	1	127	83.5%
	Pakistan	1	492	48.0%
	Oceania	**2**	**260**	**90.4%**
	New Zealand	1	230	89.1%
	Australia	1	30	100.0%
poultry by-products	**Total average**	**18**	**667**	**69.7%**
	Trinidad and Tobago	1	188	90.4%
	UK	2	17	76.5%
	Germany	1	13	23.1%
	Austria	1	44	40.9%
	Spain	1	2	100.0%
	Belarus & Russia	1	7	100.0%
	Thailand	1	16	50.0%
	Japan	9	350	60.6%
	New Zealand	1	30	100.0%
frozen poultry meats	**Total average**	**20**	**2057**	**48.5%**
	UK	7	1299	58.6%
	Germany	1	49	55.1%
	Switzerland & Liechtenstein	1	76	7.9%
	Belarus & Russia	1	28	67.9%
	Bulgaria	1	70	41.4%
	South Africa	1	33	18.2%
	Japan	7	292	24.7%
	China	1	210	37.1%
frozen poultry by-products	**Total average**	**3**	**108**	**29.6%**
ground poultry meats	**Total average**	**7**	**194**	**17.0%**

Table 2 Frequency of *Campylobacter* spp. distribution among the isolates from retail poultry in various countries

Countries	No. of references	No. of samples	Ratio			*Cc/Cj*
			C. jejuni	*C. coli*	others	(*Cj* as 100)
Argentina	1	13	100.0%	0.0%	0.0%	0.00
Belarus & Russia	1	113	100.0%	0.0%	0.0%	0.00
New Zealand	2	376	98.3%	1.7%	0.0%	1.76
Denmark	1	220	98.2%	1.8%	0.0%	1.85
Estonia	1	48	87.5%	8.3%	4.2%	9.52
Canada	3	852	90.3%	8.6%	0.6%	9.56
Japan	18	1053	89.3%	10.4%	0.3%	11.64
Belgium	1	612	87.1%	11.4%	1.5%	13.13
Ireland	2	528	86.6%	12.9%	0.6%	14.88
Barbados	1	94	79.8%	13.8%	6.4%	17.33
Australia	1	30	83.3%	16.7%	0.0%	20.00
UK	6	800	82.4%	16.8%	0.9%	20.33
Germany	6	1078	69.1%	17.9%	13.2%	25.91
Turkey	1	364	69.2%	20.9%	9.9%	30.16
Bulgaria	1	140	73.6%	26.4%	0.0%	35.92
Spain	1	51	72.5%	27.5%	0.0%	37.84
USA	4	797	64.7%	27.2%	8.0%	42.05
Pakistan	1	236	66.1%	33.9%	0.0%	51.28
Vietnam	1	31	45.2%	25.8%	29.0%	57.14
Senegal	2	373	58.7%	34.9%	6.4%	59.36
Italy	2	150	61.7%	38.3%	0.0%	62.16
Trinidad and Tobago	1	340	57.1%	42.9%	0.0%	75.26
Korea	3	1131	53.1%	46.9%	0.0%	88.50
South Africa	1	32	28.1%	37.5%	34.4%	133.33
Thailand	3	127	32.3%	54.3%	14.2%	168.29

NOTE *Public Health*

Salmonella Prevalence in Slaughtered Buffaloes and Pigs and Antimicrobial Susceptibility of Isolates in Vientiane, Lao People's Democratic Republic

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ABSTRACT. This is the first report regarding isolation of *Salmonella* from cecum samples of buffaloes and pigs and characterization of the isolates in Laos. The organisms were isolated from 8% (4/50) of buffaloes and 76% (37/49) of pigs. In buffaloes, 3 animals harbored serotype 9,12: –:1,5, and 1 animal harbored both *S.* Derby and *S.* Javiana. In pigs, the most predominant serotypes were *S.* Derby (51%) followed by *S.* Anatum (45%), *S.* Weltevreden (15%) and *S.* Stanley (5%). The buffalo isolates were susceptible to the antimicrobials tested, whereas the pig isolates showed 10 resistance patterns to 1–5 antibiotics. Of the 59 pig isolates, the resistance rates to tetracycline, streptomycin, ampicillin, sulfamethoxazole-trimethoprim, chloramphenicol, amoxicillin-clavulanic acid and nalidixic acid were 24%, 22%, 14%, 5%, 2%, 2% and 2%, respectively. The results suggest that pigs and buffaloes harbor *Salmonella*, with a higher prevalence especially in pigs, and all the isolates showed sensitivity to cefotaxime, norfloxacin and ciprofloxacin.
KEY WORDS: buffaloes, cecum contents, Lao People's Democratic Republic (Laos), *Salmonella*, swine.

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Salmonella enterica subspecies *enterica* is one of the most important foodborne bacteria in the world. Infection with *Salmonella* spp. is a major cause of diarrhea in children and young adults in developing countries [2, 17]. The reservoirs of these organisms are considered to be animals, particularly chickens and pigs, and these organisms are easily isolated from the feces [8]. The main source of infection in humans seems to be consumption of foods from animal origin such as contaminated eggs, unpasteurized milk, cross-contaminated foods and drinking water [5, 6, 9, 17]. In Southeast Asian countries, *Salmonella* spp. are commonly found in chicken eggs, chicken meat, and pork sold in markets [4, 8, 9, 11, 12].

In Lao People's Democratic Republic (Laos), there are very few reports of *Salmonella* in humans and livestock. Previous reports have suggested that the prevalence of *Salmonella* in Laotians presenting with diarrhea is only 0.6% (5/880) [16]. However, Inthavong *et al.* [10] reported that the prevalence of *Salmonella* on pig carcass swabs at slaughterhouses is relatively high (66.1%) and indicated that a large amount of contaminated meat is consumed daily. Laotians prefer to eat buffalo, pig and chicken over other meat sources; however, there are no report available concerning the prevalence of *Salmonella* in live animals or the antimicrobial susceptibility of isolates from animal feces. In the present study, we examined the prevalence of *Salmonella* in buffaloes and pigs at a slaughterhouse in Vientiane, Laos, and determined the antimicrobial susceptibility of the various isolates obtained.

Cecum swab samples were collected from 50 buffaloes and 49 pigs in February 2007 at the Dorn Du slaughterhouse in Vientiane, Laos. The buffaloes were approximately 3–10 years of age, and were usually fed roughage (not grain). The pigs were approximately 8 months of age, and were bred on personal farms. The buffaloes were shipped to the slaughterhouse from 6 livestock brokers and a few farmers, and the pigs were from 6 livestock brokers. We could not confirm the number of farms from which the pigs were shipped.

Each cecum sample was collected using 2 commercial swab sets (BD BBL Culture Swab Plus, BD, NJ, U.S.A.), stored at 4°C and immediately transported to the microbiology laboratory of the WHO International Salmonella & Shigella Center, National Institute of Health, Nonthaburi, Thailand; the samples were analyzed within 30 hr of collection. Briefly, for isolation of *Salmonella* spp., each swab sample was placed in 9 m*l* of buffered peptone water (Merck, Darmstadt, Germany), thoroughly mixed and then incubated at 37°C for 18 hr. Afterwards, 1 m*l* of pre-enrichment culture was added to 5 m*l* of Rappaport Vassiliadis (RV) broth (Merck) and incubated at 42°C for 1 day. After incubation, the RV cultures were streaked onto modified semi-solid Rappaport Vassiliadis (MSRV) agar (Merck) and Desoxycholate-Hydrogen-Sulfide-Lactose (DHL) agar (Nissui, Tokyo, Japan) and were incubated at 37°C for 18

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hr. Typical *Salmonella* colonies (1–3 colonies) were selected from each specimen for confirmation based on biochemical characteristics [7] using triple sugar iron (TSI) agar (Nissui), lysine indole motility (LIM) agar (Nissui), catalase, oxidase tests and other biochemical tests. Serotyping with anti-O and anti-H immune sera was then carried out, and the antimicrobial susceptibility of the serotypes was examined by the disk diffusion method [3] using BD Sensi-Discs (BD) with Mueller-Hinton agar plates (BD). Ten types of antimicrobial disk, namely, 10 μg of ampicillin (ABPC), 20 μg of amoxicillin with 10 μg of clavulanic acid (AMPC-CVA), 30 μg of cefotaxime (CTX), 10 μg of streptomycin (SM), 30 μg of tetracycline (TC), 30 μg of chloramphenicol (CP), 30 μg of nalidixic acid (NA), 30 μg of norfloxacin (NFLX), 5 μg of ciprofloxacin (CPFX) and 23.75 μg of sulfamethoxazole with 1.25 μg of trimethoprim (SMX-TMP) were used for the tests. In these tests, *Escherichia coli* ATCC25922, were used as quality control strains. The choice of the 10 antimicrobial disks was based on the guidelines of BD Sensi-Discs for Enterobacteriaceae (http://www.bdj.co.jp/micro/products/1f3pro00000qho5o-att/54-sd-hantei-CLSI.pdf).

As shown in Table 1, all *Salmonella* strains isolated in this study were identified as *S. enterica* subspecies *enterica*. *Salmonella* spp. were isolated from 4 (8%) of the 50 buffalo samples and 37 (76%) of the 49 pig samples. In the buffaloes, serotype 9,12: –:1,5 was isolated from 3 animals, and both *S.* Derby and *S.* Javiana were isolated from 1 animal. In the 37 positive pigs, S. Derby and *S.* Anatum were found in 12 samples, *S.* Derby and *S.* Weltevreden were found in 8 samples, *S.* Anatum was found in 8 samples, *S.* Derby was found in 5 samples, *S.* Stanley and *S.* Anatum were found in 3 samples, *S.* Stanley was found in 1 sample and *S.* Weltevreden was found in 1 sample. In the present study, the most predominant serotypes isolated were *S.* Derby (51%; 25/49), *S.* Anatum (45%; 22/49), *S.* Weltevreden (15%; 9/49) and *S.* Stanley (5%; 3/49).

We examined the antimicrobial resistance of 5 isolates from the 4 buffaloes and 59 isolates from the 37 pigs. All the isolates from the buffaloes were susceptible to the 10 antimicrobials tested. Table 2 shows the proportion of *Salmonella* isolates from the pigs demonstrating antimicrobial resistance by serotype. The resistance rates to ABPC, AMPC-CVA, SM, TC, CP, NA and SMX-TMP of the pig isolates were 14%, 2%, 22%, 24%, 5%, 2% and 12%, respectively; all the isolates were susceptible to CTX, NFLX and CPFX.

Table 3 shows the profile of antimicrobial resistance of the *Salmonella* isolates from the pigs. Among the 21 resistant isolates, there were 8 multidrug resistance patterns, and the ABPC/SM/TC/CP/SMX-TMP pattern was the most frequent (3 strains; all S. Derby). Monodrug resistance to SM

Table 1. Prevalence of *Salmonella* spp. in cecum samples obtained from buffaloes and pigs at a slaughterhouse in Lao PDR

Animal species	No. of animals	No. of positive samples (%)	Serotype	No. of samples[a)]
Buffalo	50	4(8)	9,12: –:1,5	3
			Derby and Javiana	1
Pig	49	37 (76)	Derby and Anatum	12
			Derby and Weltevreden	8
			Anatum only	8
			Derby only	5
			Stanley and Anatum	2
			Stanley only	1
			Weltevreden only	1

a) *Salmonella* 9,12:–:1,5, *S.* Derby and *S.* Javiana were isolated from 6% (3/50), 2% (1/50) and 2% of the buffaloes (1/50), respectively. *S.* Derby, *S.* Anatum, *S.* Weltevreden and *S.* Stanley were isolated from 51% (25/49), 45% (22/49), 18% (9/49) and 6% of the pigs (3/49), respectively.

Table 2. Proportion of *Salmonella* isolates from pigs demonstrating antimicrobial resistance by serotype

Serotype	No. of strains examined	Antimicrobial agents[a)]									
		ABPC	AMPC-CVA	CTX	SM	TC	CP	NA	NFLX	CPFX	SMX-TMP
Derby	25	20	4		36	20	12	4			12
Anatum	22	14			9	27					14
Weltevreden	9										
Stanley	3				67	67					33
Total	59	14	2		22	24	5	2			12

a) ABPC: ampicillin. AMPC-CVA: amoxicillin with clavulanic acid. CTX: cefotaxime. SM: streptomycin. TC: tetracycline. CP: chloramphenicol. NA: nalidixic acid. NFLX: norfloxacin. CPFX: ciprofloxacin. SMX-TMP: sulfamethoxazole with trimethoprim.

Table 3. Numbers of *Salmonella* isolates from pigs with different resistance profiles

Profiles[a)]	No. of isolates	*Salmonella* serotype			
		Derby	Anatum	Weltevreden	Stanley
Noresistance demonstrated	38	12	16	9	1
Resistance to one agent					
TC only	3	1	2		
SM only	6	6			
Total	9	7	2	0	0
Resistance to two agents					
ABPC/AMPC-CVA	1	1			
ABPC/TC	1	1			
SM/TC	2		1		1
TC/NA	1	1			
Total	5	3	1	0	1
Resistance to three agents					
ABPC/TC/SMX-TMP	2		2		
SM/TC/SMX-TMP	1				1
Total	3	0	2	0	1
Resistance to four agents					
ABPC/SM/TC/SMX-TMP	1	0	1	0	0
Resistance to five agents					
ABPC/SM/TC/CP/SMX-TMP	3	3	0	0	0
Total	59	25	22	9	3

a) ABPC: ampicillin. AMPC-CVA: amoxicillin with clavulanic acid. CTX: cefotaxime. SM: streptomycin. TC: tetracycline. CP: chloramphenicol. NA: nalidixic acid. NFLX: norfloxacin. CPFX: ciprofloxacin. SMX-TMP: sulfamethoxazole with trimethoprim.

(6 strains; all *S.* Derby) and TC (3 strains; 2 strains of *S.* Anatum and 1 strain of *S.* Derby) was predominant in the isolates from pigs. Multiantimicrobial resistance was shown by *S.* Derby, *S.* Anatum and *S.* Stanley.

In the present study, the prevalence of *Salmonella* in the buffaloes was 8% (4/50), and the most predominant serotype identified was 9,12:–:1,5. To the best of our knowledge, there are no reports available concerning the prevalence of *Salmonella* in buffaloes in Asia. The prevalence of *Salmonella* among domestic buffaloes in Laos may generally be around 8%, and serotype 9,12:–:1,5 may particularly be observed in buffaloes in Laos.

The prevalence of *Salmonella* in the pigs was 76% (37/49), and the most predominant serotypes identified were *S.* Derby and *S.* Anatum. The prevalence and serotypes of the isolates in the pigs were thought to depend on geographic location; for example, in a previous study, 12% (7/110 heads) of slaughtered pigs in Gunma Prefecture, Japan, harbored *Salmonella*, and the predominant serotypes were *S.* Typhimurium and *S.* Derby [13]. On the other hand, in a study conducted in Vietnam, *Salmonella* spp. were isolated from 5% (23/439 heads) of pigs, and the predominant serotypes were *S.* Javiana (9/25 strains), *S.* Derby(4/25 strains) and *S.* Weltevreden (3/25 strains) [15]. In a study conducted in Northern Thailand, the overall prevalence of *Salmonella* in pigs in slaughterhouses was 28% (97/349 strains), and most of the serotypes isolated were *S.* Rissen, *S.* Weltevreden and *S.* Anatum [11]. The prevalence of *Salmonella* in Laos obtained in the present study was higher than that in other Asian countries. We could not, however, confirm the number of farms from which the pigs were shipped by the 6 livestock brokers, and the present study was conducted in only 1 slaughterhouse. In Laos, large-scale commercial pig production is not common and therefore many pigs are bred on personal farms. In this regard, additional surveys of different pig farms are necessary. *S.* Derby and *S.* Anatum are the most common serotypes found in animals and humans throughout the world; however, *S.* Weltevreden has recently been reported to be a frequent and increasing cause of human salmonellosis and is the predominant serotype in Southeast Asian countries [1, 4, 14, 15]. The results of the present study indicate that *S.* Weltevreden might be widely distributed in pigs in Laos.

In general, high sensitivity to most antimicrobial agents was observed among the buffalo and pig isolates. In Laos, the use of antimicrobial agents as a feed additive to animals is not regulated; however, nobody gives feed additives to animals because most pigs and buffaloes are bred on personal farms. The unpopularity of using antimicrobial agents as feed additives may be one of the predisposing factors for many *Salmonella* strains with high sensitivity to antimicrobial agents.

The cecum contents of pigs and buffaloes may be potential vehicles for contaminating edible products at slaughter-

houses. A survey of the prevalence of *Salmonella* spp. in other food animals, such as chickens, and other foods should be performed to determine the important sources of *Salmonella* infection in Laos.

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NOTE ***Public Health***

Prevalence of *Campylobacter* spp. in Slaughtered Cattle and Buffaloes in Vientiane, Lao People's Democratic Republic

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ABSTRACT. This is the first report regarding isolation of *Campylobacter* in caecum and bile samples obtained from ruminants in Vientiane, Lao PDR. *Campylobacter* was isolated from 3 (1.6%) of the 184 caecum samples and 1 (1.0%) of the 100 bile samples obtained from buffaloes. Three of the 4 isolates were determined to be *C. jejuni*, which was detected in 2 caecum samples and 1 bile sample; the other caecum sample contained *C. fetus*. *Campylobacter* was not isolated from any of the 82 cattle caecum samples. Our results suggest that cattle and buffaloes may not be important sources of *Campylobacter* food poisoning in Lao PDR.

KEY WORDS: *Campylobacter*, Lao People's Democratic Republic (PDR), ruminant.

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Campylobacter spp. are gram-negative bacteria belonging to the family *Campylobacteraceae*, which includes bacteria with a microaerobic growth requirement. The intestine is the reservoir for *Campylobacter* spp. in animals, especially in chickens and cattle [23]. These organisms are easily isolated from the feces and/or bile of chickens and ruminants [2, 4, 15, 21, 22]. Infection with *Campylobacter* spp. has been reported to be a major cause of illness in children and young adults in developing countries [12, 20, 26]. In particular, it has been isolated from the stool of diarrheal patients and animals and from contaminated food and water [1, 9, 13, 16, 17]. The main reason for occurrence of this infection in humans is considered to be consumption of food of animal origin cross-contaminated food or drinking water, and unpasteurized milk [6, 7, 13, 27].

Previous reports have suggested that the prevalence of *C. jejuni* in children in Southeast Asian countries varies from 2.9% to 15% [3, 16, 20, 26]; in particular, 2.9% to 4.4% of Laotian children with diarrhea are infected with this organism [18, 29]. Chicken is the most important source of *Campylobacter* food poisoning worldwide [23]. However, commercial, large-scale chicken production is not carried out in Lao People's Democratic Republic (Lao PDR). Instead, chickens are bred in farmhouses. Laotians prefer to eat beef, particularly from buffaloes, pork and chicken. In addition, cases of avian influenza were reported during the sampling period of this study. Consumption of beef is also a source of *Campylobacter* food poisoning because a high prevalence of *Campylobacter* spp. has been recognized in caecum and bile samples from cattle [4, 5, 8–11, 14–17, 21–23, 27, 28]. The present study was the first survey of the prevalence of *Campylobacter* spp. in cattle and buffaloes at a slaughterhouse in Vientiane, Lao PDR, conducted to determine whether cattle are a source of *Campylobacter* infection.

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Study samples were collected on a total of 6 days between March and September 2006 (once per month, excluding June) at the Dorn Du slaughterhouse in Vientiane, Lao PDR. A large number of the ruminants were roughage-fed (not grain-fed). Each day, 15–20 cattle and 30–40 buffaloes are slaughtered in the slaughterhouse. The ruminants are approximately 3–10 years of age. The samples used in the present study consisted of 82 caecum samples collected from the feces of cattle and 184 caecum and 100 bile samples collected from buffaloes. These cattle and buffaloes were shipped to the slaughterhouse from 4 livestock brokers. We could not confirm the number of farms from which the cattle and buffaloes were shipped.

The caecum samples (approximately 1 g) were collected using a disposable dropping pipette and were stored in 9 m*l* of semisolid Cary-Blair transport medium prepared by us. The bile samples were collected using disposable syringes, stored at 4°C, and immediately transported to the laboratory of the Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand. The stored samples were analyzed within 30 hr of collection. Isolation and identification of *Campylobacter* spp. were performed in accordance with the guidelines of Public Health Laboratory Services (PHLS) [19]. Briefly, a caecum sample in semisolid Cary-Blair transport medium (1 m*l*; caecum volume = 0.1 m*l*) or bile sample (1 m*l*) was added to 9 m*l* Preston *Campylobacter* selective enrichment broth. This broth is comprised of brucella broth supplemented with Preston *Campylobacter* selective supplement SR 0117E, *Campylobacter* growth supplement SR 0232E (Oxoid, Hampshire, UK), and 5% defibrinated horse blood. Next, the mixture was incubated

at 42°C for 1 day under microaerophilic conditions consisting of 5% O_2, 10% CO_2, and 85% N_2 using a GasPak jar system (Mitsubishi Chemical Corporation, Tokyo, Japan). After incubation, the specimens were streaked onto modified CCDA-Preston (mCCDA) consisting of a *Campylobacter* blood-free selective agar base (Oxoid) supplemented with *Campylobacter* CCDA selective supplement SR 0155E (Oxoid) and were incubated at 42°C for 3–5 days under microaerophilic conditions. A typical *Campylobacter* colony was selected from each specimen for identification of the strains by gram staining and biochemical tests, such as the catalase, oxidase, and hippurate hydrolysis tests.

As shown in Table 1, *Campylobacter* spp. were isolated from 3 (1.6%) of the 184 caecum samples and 1 (1.0%) of the 100 bile samples from the buffaloes. No *Campylobacter* spp. were isolated from the cattle caecum samples. *C. jejuni* was detected in the caecum samples collected from two buffaloes in March and September and in a bile sample from one buffalo collected in September. None of the bile and caecum samples from the same animal tested positive for *C. jejuni*. *C. fetus* was isolated from one buffalo caecum sample collected in September. Furthermore, multiple *Campylobacter* spp. were not isolated from any one animal.

Numerous reports from Europe, Asia, and the US have described isolation of *Campylobacter* spp. from the fecal and bile samples of cattle [4, 5, 8–11, 14–17, 21–23, 27, 28]. The rates of *Campylobacter* spp. isolation from the fecal and bile samples of ruminants in these reports are relatively high, i.e., 4%–91% from fecal samples and 16%–97% from bile samples. Variables such as herd size; type, season, and age of the animal; sample site; sample frequency; isolation method; geography; and diet and husbandry practices have been suggested as reasons for the differences in rates of isolation [14, 24, 25]. Our present data demonstrated that only 1.6% of the caecum samples and 1.0% of the bile samples from the buffaloes were positive for *C. jejuni* and *C. fetus*; no *Campylobacter* spp. were isolated from the cattle caecum samples. The prevalence of *Campylobacter* spp. in cattle and buffaloes was relatively low in this study. Wesley *et al.* [28] suggested that *C. jejuni* is more frequently isolated from herds fed alfalfa, whole cottonseed, or a hull diet. This suggests that a diet of roughage may be one of the reasons for the low prevalence of *Campylobacter* spp. in ruminants and that these animals may not be an important source of *Campylobacter* food poisoning in Lao PDR.

According to the report of Yamashiro *et al.* [29], *Campylobacter* spp. are isolated at a relatively low frequency (only 4.4%) from Laotian patients with diarrhea. A survey of the prevalence of *Campylobacter* spp. in other food animals, such as chicken and swine, should be performed to determine significant sources of *Campylobacter* infection in Lao PDR.

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Table 1. Prevalence of *Campylobacter* spp. in caecum and bile samples obtained from ruminants at a slaughterhouse in Lao PDR

Animal species	Sample	Number	No. of positive samples (%)	Species	No. of samples
Cattle	Caecum	82	0		
Buffaloes	Caecum	184	3 (1.6)[a]	*C. jejuni*	2
				C. fetus	1
	Bile	100	1 (1.0)	*C. jejuni*	1

a) None of the animals harbored *C. jejuni* in both bile and caecum samples. Multiple *Campylobacter* spp. were not isolated from any one animal.

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FULL PAPER *Public Health*

Isolation of *Mycobacterium* spp. from Milking Buffaloes and Cattle in Nepal

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ABSTRACT. In Nepal, mycobacterial isolates obtained from the milk and feces of buffaloes and cattle that were positive for the single intradermal cervical tuberculin (SICT) tests were genetically identified. A total of 36 mycobacterial strains were isolated from 39% of the buffaloes (14 of 36) and 34% of the cattle (11 of 32). Of the 36 strains, 13 were identified as *M. bovis*, and these strains were isolated from 17% of the buffaloes (6 of 36) and 16% of the cattle (5 of 32). *M. bovis* was isolated from both the milk and feces of one buffalo and one cattle, the milk alone of three buffaloes and three cattle, and the feces alone of two buffaloes and one cattle. These results suggest that milking buffaloes and cattle infected with *M. bovis* exist in Nepal. The remaining 23 strains were atypical mycobacteria. A program for the elimination of bovine tuberculosis should be implemented as soon as possible, and the public health education and proper hygienic practices may be required.

KEY WORDS: buffalo, cattle, isolation, mycobacteria, Nepal.

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In humans and animals, mycobacteria such as those belonging to the *Mycobacterium tuberculosis* complex (*M. tuberculosis, M. bovis, M. africanum, M. canettii*, and *M. microti*) cause serious diseases, and atypical mycobacteria cause opportunistic infections. Many species of mycobacteria have been identified and it has been suggested that many unknown atypical mycobacteria species exist [10, 20, 24]. In developing countries, *M. tuberculosis* is one of the most common causes of human tuberculosis. In humans, however, *M. bovis* infection exists and a large population of unknown causes may be attributable to *M. bovis* [2]. In addition, ruminant animals such as cattle and buffaloes are thought to be the reservoirs of *M. bovis* [2, 3, 5, 9, 18, 19]. Previously, we had examined the results of the single intradermal cervical tuberculin (SICT) test was carried out by injection tuberculin into the skin of the neck of a large number of cattle and buffaloes in Nepal. We found that approximately 5% of these animals had a history of *M. bovis* infection, and that food products from these animals were being consumed daily in the country [21]. However, this study did not determine the *M. bovis* strain that was responsible for the infection.

Recently, mycobacterial species were genetically identified by using *rpoB* gene analysis [10, 11]. In addition, *M. tuberculosis* and *M. bovis* were identified by using the hypothetical protein "Rv1506c" [1]. In this study, we applied a genetic method such as sequence homology of the *rpoB* gene to the identification of the species of the isolates, and investigated the prevalence of mycobacteria in the milk and feces of SICT test-positive milking buffaloes and cattle. We also performed sequence and phylogenetic analyses of their *rpoB* gene.

MATERIALS AND METHODS

Samples: From September to October in 2003, we collected the milk and fecal samples from 36 buffaloes and 32 cattle that were SICT test-positive. The animals were selected from the Kathmandu district and the 6 dairy pocket areas such as Shishuwa, Dhikurpokhari, Chorepatan, Hemja, Pumdi Bhumdi, and Armala of the Kaski district, Nepal. We collected the milk samples while farmers were milking the animals in farms and the fresh fecal samples from the rectum of the animals. The milk was for consumption by the Nepalese people. The samples were placed in sterile tubes, stored at 4°C, and brought to the Regional Veterinary Laboratory in Pokhara within 5 days after collection.

Isolation procedures: Milk sample (10 m*l*) was centrifuged at 1,900G for 20 min. We discarded the supernatant and added 10 m*l* of 7H9 liquid culture medium (Oxoid, Hampshire, England) to the sediment and mixed it vigorously for 10 s. The growth culture was incubated at 37°C for 1 to 2 days. Fecal sample (1 g) was inoculated into 10 m*l* of 0.1% acriflavin solution, mixed well, and centrifuged at 1,900G for 20 min. After discarding the supernatant, 10 m*l* of 4% NaOH solution was added to the sediment. The samples were mixed well and left standing for 15 min. A volume of 0.1 m*l* of the 7H9 liquid culture sample (milk) or alkaline-treated sample (fecal sample) was streaked onto each of 2 Tween egg media [23] prepared in the Nepal labo-

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ratory by us. The inoculated medium was incubated for 30 to 60 days at 37°C under aerobic conditions. During the incubation period, we observed the slant media daily, and all presumptive mycobacterial colonies growing on the media were observed, and subcultured onto new Tween egg medium for 30 to 60 days at 37°C under aerobic conditions. Colonies growing on the medium were selected, being based on their morphology determined by Ziehl-Neelsen staining.

Statistical analysis: A chi-square test was used to compare the mycobacteria isolating rates between buffaloes and cattle. Differences were considered significant when the p values were less than 0.05.

DNA extraction, PCR amplification, amplicon sequencing and identification of mycobacterial species: In order to generate templates for PCR, we purified the DNA obtained from the strains by the boiling method (incubating the strains at 100°C for 10 min) in Nepal. For the identification of the mycobacterial species, we performed a comparative sequence analysis of the RNA polymerase gene (*rpoB*) [10] in Japan. For the amplification of the *rpoB* gene, we used a set of previously reported primers: MF primer (5' CGA CCA CTT CGG CAA CCG 3') and MR primer (5' TCG ATC GGG CAC ATC CGG 3') (theoretical amplicon of 342 nt) [10]. The PCR reaction mixture contained 1 μl of template DNA, 1 μl of the primers MF and MR (20 pmol each), 12.5 μl of PCR Master Mix (Promega, Madison, M.I., U. S. A.), and 9.5 μl of distilled water free from DNase and RNase (total volume, 25 μl). The PCR protocol included incubation for 3 min at 95°C followed by 30 cycles at 95°C for 30 s, at 60°C for 30 s, at 72°C for 45 s, and an additional 5 min for elongation at 72°C after the last cycle. The sizes of the amplified DNA fragments were confirmed by electrophoresis on a 1.5% agarose gel. After purification of the DNA fragments with a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany), the 342-bp nucleotide sequence that included the *rpöB* gene was determined by using an automated DNA sequencer (ABI 310 DNA sequencer, Applied Biosystems, Foster City, CA., U.S.A.) and a Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) [14]. Nucleotide sequences (306 bp) of the amplicon (342 bp) were analyzed genetically using the BLAST program (http://www.ddbj.nig.ac.jp/search/blast-e.html) and the CLUSTAL W program (http://www.ddbj.nig.ac.jp/search/clustalw-e.html) provided by the DNA Data Bank of Japan (DDBJ) home page. At the BLAST program, the isolates having more than 99% similarity with reference strains registered (GenBank no. AF060279 to AF060367) by Kim *et al.* (1999) [10] were genetically identified as mycobacterial species, as shown in Fig. 1. At the CLUSTAL W program, evolutionary distances were estimated by using Kimura's 2-parameter method [13], and the phylogenic trees were constructed using the neighbor joining (NJ) method [22]. The reliability of the tree was estimated by using 1,000 bootstrap replications.

Identification of M. bovis from the M. tuberculosis complex using the hypothetical protein "Rv1506c" that was a 12.7-kb fragment: For the differentiation of *M. bovis* from the *M. tuberculosis* complex, we performed multiplex PCR targeting hypothetical protein "Rv1506c" that was a 12.7-kb fragment [1] in Japan. Oligonucleotide primer sequences used in the PCR method included a common forward primer; CSB1 (5' TTC CGA ATC CCT TGT GA 3') and two reverse primers, including the *M. bovis*-specific CSB2 (5' GGA GAG CGC CGT TGT A 3'), and the *M. tuberculosis*-specific CSB3 (5' AGT CGC CGT GGC TTC TCT TTT A 3'). The PCR reaction mixture contained 1 μl of the template DNA, 1 μl of the primers CSB1, CSB2, and CSB3 (25 pmol each), 12.5 μl of PCR Master Mix (Promega), and 8.5 μl of distilled water free from DNase and RNase (total volume, 25 μl). The PCR protocol included incubation for 2 min at 94°C followed by 30 cycles at 94°C for 60 s, at 52°C for 90 s, at 72°C for 60 s, and an additional 5 min for elongation at 72°C after the last cycle. The amplification products were analyzed by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide fluorescence. The amplification product that was either 168 bp (*M. bovis*-specific) or 263 bp (*M. tuberculosis*-specific) must be visualized using the DNA obtained from the respective species. Subsequently, we sequenced the amplification product by using an automated DNA sequencer and a Dye Terminator Cycle Sequencing Ready Reaction Kit.

RESULTS

Isolation and identification of mycobacterial species: As shown in Table 1, Mycobacterial strain was isolated from 39% of the buffaloes (14 of 36) and 34% of the cattle (11 of 32); *M. bovis* was isolated from 17% of the buffaloes (6 of 36) and 16% of the cattle (5 of 32). There was no statistical significance with regard to the difference in the percentages of the buffaloes and cattle isolating Mycobacteria and *M. bovis* (chi square test, $p<0.05$). *M. bovis* was isolated from the animals kept at farms in the pocket areas of the Kaski district, including Shishuwa (7 head), Dhikurpokhari (2 head), Hemja (1 head), and Pumdi Bhumdi (1 head). *M. bovis* strains were not isolated from the SICT test-positive animals kept in Kathmandu and the 2 pocket areas of Chorepatan and Armala in the Kaski district.

As shown in Table 2, a total of 36 mycobacterial strains were isolated from 14 buffaloes and 11 cattle. By using the sequence homology of the *rpoB* gene present in the isolates, 28 of 36 strains could be identified as 8 mycobacterial species, including the *M. tuberculosis* complex (*M. bovis*; 13 strains), *M. thermoresistibile* (6 strains), *M. xenopi* (2 strains), *M. fortuitum* (2 strains), *M. chelonae* (2 strains), *M. gordonae* (1 strain), *M. ulcerans* (1 strain), and *M. kansasii* (1 strain), while 8 strains could not be identified. *M. bovis* was isolated from the milk of 8 animals (B-4, B-6, B-10, B-12, C-5, C-8, C-10, and C-11) and the feces of 5 animals (B-6, B-7, B-13, C-4, and C-8). Of these, 2 animals (B-6 and C-8) had *M. bovis* in both milk and feces. In addition, a total of 23 atypical mycobacteria strains were isolated from 16

Table 1. Isolation of *Mycobacterium* organisms from SICT tests positive- buffaloes and cattle in the Kathmandu and Kaski districts, Nepal

District, pocket area	Buffaloes: heads			Cows : heads			Total : heads		
	SICT tests-positive	Mycobacteria isolated (%)	*M. bovis* isolated (%)	SICT tests-positive	Mycobacteria isolated (%)	*M. bovis* isolated (%)	SICT tests-positive	Mycobacteria isolated (%)	*M. bovis* isolated (%)
Kathmandu	9	3 (33)	0 (0)	14	3 (21)	0 (0)	23	6 (26)	0 (0)
Kasuki district									
Shishuwa	19	7 (37)	4 (21)	10	5 (50)	3 (30)	29	12 (41)	7 (24)
Dhikurpokhari	5	3 (60)	2 (40)				5	3 (60)	2 (40)
Chorepatan	1	1 (100)	0 (0)	4	1 (25)	0 (0)	5	2 (40)	0 (0)
Hemja				2	1 (50)	1 (50)	2	1 (50)	1 (50)
Pumdi Bhumdi	2	0 (0)	0 (0)	1	1 (100)	1 (100)	3	1 (33)	1 (33)
Armala				1	0 (0)		1	0 (0)	0 (0)
Total	36	14 (39)[a]	6 (17)[b]	32	11 (34)[a]	5 (16)[b]	68	25 (37)	11 (16)

a, b) There was no statistical significance with regard to the difference in the percentages of the buffaloes and cows isolating mycobacteria (chi square test, $p<0.05$).

Table 2. History of strains examined in this study, species name, strain number, and GenBank accession number

District, pocket area		Animal no.[a]	Sample	Species or strain	Strain no.	GenBank accession no.[b]
Kathmandu		B-1	feces	*M. xenopi*	1	AB244246
		B-2	feces	*M. thermoresistibile*	2	AB244247
		B-3	feces	*M. gordonae*	3	AB244248
		C-1	milk	*M. xenopi*	4	AB244249
		C-2	feces	*M. ulcerans*	5	AB244250
		C-3	feces	*M.* spp., Nepal-3	6	AB126347
Kaski	Shishuwa	B-4	milk	*M. bovis*	7	AB170004
		B-5	milk	*M.* spp., Nepal-4	8	AB170008
		B-6	milk	*M. bovis*	9	AB244251
			feces	*M. bovis*	10	AB244252
			milk	*M. kansasii*	11	AB244253
		B-7	feces	*M. bovis*	12	AB244254
		B-8	feces	*M. thermoresistibile*	13	AB170007
			milk	*M.* spp., Nepal-5	14	AB244255
		B-9	feces	*M. thermoresistibile*	15	AB244256
		B-10	milk	*M. chelonae*	16	AB244257
			milk	*M. bovis*	17	AB244258
		C-4	feces	*M. bovis*	18	AB244259
			milk	*M.* spp., Nepal-1	19	AB244260
		C-5	milk	*M. bovis*	20	AB244261
		C-6	feces	*M.* spp., Nepal-4	21	AB244262
		C-7	feces	*M. thermoresistibile*	22	AB244263
		C-8	milk	*M. bovis*	23	AB244264
			feces	*M. bovis*	24	AB244265
	Dhikurpokhari	B-11	milk	*M.* spp, Nepal-1	25	AB244266
			feces	*M. thermoresistibile*	26	AB244267
			feces	*M.* spp., Nepal-1	27	AB126345
			feces	*M.* spp., Nepal-2	28	AB126346
		B-12	milk	*M. bovis*	29	AB244268
			feces	*M. fortuitum*	30	AB244269
		B-13	feces	*M. bovis*	31	AB244270
			feces	*M. chelonae*	32	AB244271
	Chorepatan	B-14	milk	*M. fortuitum*	33	AB170005
		C-9	milk	*M. thermoresistibile*	34	AB170006
	Hemja	C-10	milk	*M. bovis*	35	AB244272
	Pumdi Bhumdi	C-11	milk	*M. bovis*	36	AB170003

a) B and C is the animal number designated buffaloes and cattle, respectively.
b) GenBank accession numbers refer to the *rpoB* sequences of the reference strains.

animals (B-1, B-2, B-3, B-5, B-6, B-8, B-9, B-10, B-11, B-12, B-13, B-14, C-4, C-6, C-7, and C-9), and several mycobacterial species were isolated from 7 animals (B-6, B-8, B-10, B-11, B-12, B-13, and C-4).

Phylogenetic analysis based on the sequences of the rpoB gene: The phylogenetic trees constructed by the NJ method are shown in Fig. 1. The tree was clearly divided into 2 clusters of rapid growing (Cluster A) and slow growing (Cluster B) mycobacteria. The measures of genetic diversity in the 2 clusters were approximately 0.09 (Cluster A) and approximately 0.08 (Cluster B). The *rpoB* sequences of the 13 *M. bovis* strains (strain nos. 7, 9, 10, 12, 17, 18, 20, 23, 24, 29, 31, 35, and 36) exactly matched those of *M. tuberculosis* (GenBank Accession no., X06422), *M. africanum* (AF057450), and *M. bovis* (AF057451 and AF057555). The species of 15 atypical mycobacteria strains could be identified because the *rpoB* gene of these isolates showed more than 99% sequence similarity with that of the reference strains used in the BLAST program based on previously report by Kim *et al.* (1999)[10]. We could not determine the species of 8 strains because homology of the gene to the reference strain was between 96% and 98%; the Nepal-1 (GenBank Accession no. AB126345) to *M. nonchromogenicum* (AF057478) was 98%, the Nepal-2 (AB126346) to *M. fortuitum* (AF057464) was 97%, the Nepal-3 (AB126347) to *M. senegalense* (AF057483) was 96%, the Nepal-4 (AB170008) to *M. senegalense* (AF057483) was 96%, and the Nepal-5 (AB244256) to *M. thermoresistibile* (AF057489) was 98%. The mycobacterial species Nepal-1 belonged to cluster B, and Nepal-2 to -5 belonged to cluster A. The following species were grouped together in the same subcluster: Nepal-1 and *M. nonchromogenicum*, Nepal-5 and *M. thermoresistibile*, and Nepal-2 to -4 and *M. senegalense*. The *rpoB* gene of strain nos. 19, 25, and 27, which belong to Nepal-1, and that of strain nos. 8 and 21, which belong to Nepal-4, were identical.

DISCUSSION

M. bovis has extremely high virulence to cattle because a single colony-forming unit of *M. bovis* via air can result in tuberculosis infection in cattle [7], and the organism belongs to zoonotic bacteria [2, 3]. Control and/or eradication of bovine tuberculosis in livestock animals have been being carried out in the world. For control of the disease, tuberculin tests have been frequently used for the diagnosis of tuberculosis in cattle, and the sensitivity and specificity of the test are reliable for more than 100 years [6, 16]. Cornejo *et al.* [4] suggested that *M. bovis* was detected from approximately 24% of the milk of cattle with a positive tuberculin skin test. In this study, we found that isolation rate of *M. bovis* between milking buffaloes and cattle was no different statistically, and the organism was isolated from the milk and/or feces of approximately 16% SICT test-positive milking buffaloes and cattle. Cornejo *et al.* [4] also reported that approximately 94% of milk samples obtained from tuberculin skin test positive cattle were harboring gene of *M. bovis*, and the gene were detected by PCR method. Although tuberculin skin test has been shown to display both false-positive and false-negative results, the main reason of low isolation rate of *M. bovis* in milk from the animals was sensitivity deficiency in the isolation method used in this study. However, approximately 16% of the SICT test-positive milking buffaloes and cattle were excreting *M. bovis* into the environment, and the humans as well as other animals may possibly take the organisms. Our results suggested that eradication program against the SICT test-positive animals have to need based on veterinary, food and medical hygiene.

The main lesions in cattle infected bovine tuberculosis are found in the upper/lower respiratory tracts associated with the lymph nodes and mesenteric lymph nodes [3]. In this study, we did not perform autopsies of the SICT test-positive animals, and the detailed studies including the pathology of *M. bovis* infection are yet to be performed. In addition, we could not completely rule out the external contamination of the milk samples during sampling because we collected the samples while the farm workers were milking the animals. A large amount of insufficiently pasteurized milk and dairy products are consumed daily in Nepal [8]. It has been reported that dairy products are contaminated with various pathogens by the pathogen-carrying animals, and these contaminated foods may cause various infections in humans [2,15]. According to the World Health Organization (WHO) report about tuberculosis in Nepal (http://www.who.int/inf-new/tuber4.htm), almost half of the 20 million population are infected with tuberculosis. Of these, approximately 90,000 people have active tuberculosis and there are 44,000 new cases occurring every year. There is no research done about the prevalence of *M. bovis* infection in human tuberculosis in Nepal. However, we suppose that more than a few cases caused by *M. bovis* may exist. Thus, it is possible that *M. bovis* infections may be transmitted to Nepali people via dairy products.

Atypical mycobacteria exist in our environment, and most of them are non-pathogenic to animals and humans, excluding avian tuberculosis caused by *M. avium* in birds [3, 24]. Some mycobacterial species produce reactor animals for the tuberculin test with no visible lesions [16, 20, 24]. However, such reactions occurred in a small population only [16]. In our study, seven species of atypical mycobacteria such as *M. thermoresistibile*, *M. xenopi*, *M. fortuitum*, *M. chelonae*, *M. gordonae*, *M. ulcerans*, and *M. kansasii*, and the 5 types of unidentified mycobacteria were isolated from the SICT test-positive animals. To have a better understanding about the isolates concerning pathogenic or non-pathogenic to the animals, pathological and serological studies are required.

Molecular typing methods based on sequence analysis in *Mycobacterium* have been recently developed [10,12,17], and it allows us to identify the various mycobacterial genera or species. In addition, culturing is not useful to identify the species because many pathogenic mycobacteria such as *M. tuberculosis* complex belong to slow growing mycobacteria. Therefore, we applied a genetic method such as sequence

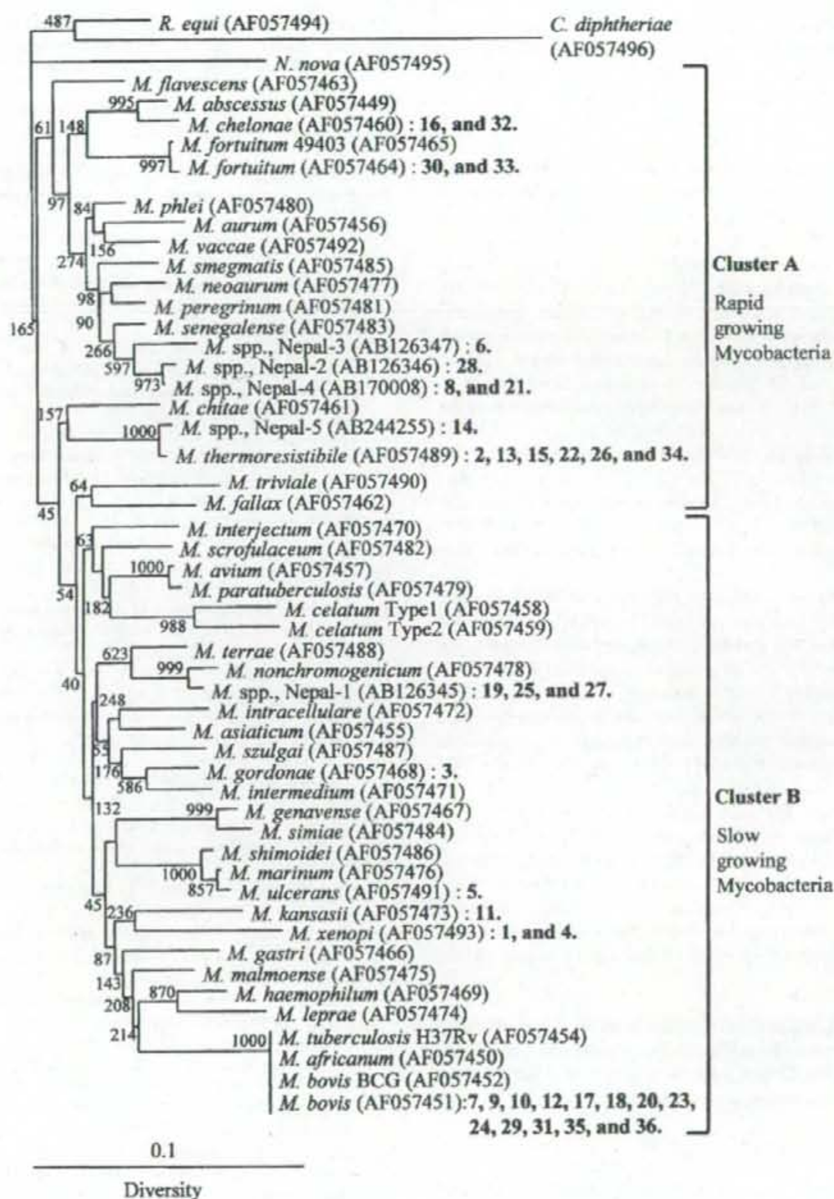

Fig. 1. Phylogenetic rooted trees of the *rpoB* gene constructed by the neighbor joining method. The methods for phylogenetic analysis and the identification of the strains using these trees are described in detail in the text. Numbers at each branch indicate bootstrap values for the clusters supported by that branch. *Rhodococcus equi* (GenBank accession no. AF057494), *Nocardia nova* (AF057495), and *Corynebacterium diphtheriae* (AF057496) were determined simultaneously. GenBank accession numbers of the reference strains used in this study have been mentioned in parentheses. The strain numbers of the isolates used in this study are designated by bold-type numerals and their GenBank accession numbers are indicated in Table 2.

homology of the *rpoB* gene to identification of the species of the isolates derived from the ruminants, and 28 of 36 strains could be identified as 8 mycobacterial species. In addition, we performed multiplex PCR targeting hypothetical protein "Rv1506c" for the identification of *M. bovis* from the *M. tuberculosis* complex. Nowadays, transport of live pathogenic bacteria between countries is restricted for the animal hygiene and security, and thus genetic method without handling live bacteria using comparative sequence analysis of the amplified *rpoB* gene and multiplex PCR targeting "Rv1506c" are useful to identify the mycobacteria. In fact, we isolated mycobacteria and purified the DNA from isolates by the boiling method in Nepal, and then transferred the DNA without live bacteria to Japan under the approval of the Nepali and the Japanese governments in this study.

We isolated 13 strains of *M. bovis*, and the *rpoB* sequences of these strains completely matched those of the *M. tuberculosis* complex, including *M. tuberculosis* (GenBank Accession no. X06422), *M. africanum* (AF057450), and *M. bovis* (AF057451, and AF057555). The results suggested that the nucleotide sequences of the *rpoB* gene in various strains of the *M. tuberculosis* complex may be highly conserved. This was consistent with the previous report [10].

Nepali farmers continue to keep many SICT test-positive milking buffaloes and cattle [21], and *M. bovis* is still present in the milk and feces of some of these animals. *M. bovis* can survive for long periods outside an animal host, and it is a highly infective pathogen in animals as well as humans. The WHO recommends the implementation of a test-and-slaughter policy to eliminate bovine tuberculosis from the country or region [5]. However, since Hinduism is widely practiced in Nepal, slaughtering of cattle is forbidden. Programs for the eradication of bovine tuberculosis, such as the identification of animals infected with the disease using the SICT test, an effective monitoring of animal movements, and a nation-wide campaign for a standardized procedures for pasteurization of milk should be implemented as soon as possible. Additionally, public health education and proper hygienic practices need to be popularized in Nepal.

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Serotypes, Antimicrobial Susceptibility, and *gyr A* Gene Mutation of *Campylobacter jejuni* Isolates from Humans and Chickens in Thailand

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***Abstract*: In Thailand, 51% (36/70) *Campylobacter jejuni* isolates from humans and 68% (47/69) isolates from poultry were classified into 10 Penner serotypes (serotype B, C, R, E, G, A, K, D, I, and L) and 9 serotypes (serotype A, C, I, K, B, E, S, D, and L), respectively. The rate of antimicrobial drug resistance to nalidixic acid, ciprofloxacin, ampicillin, tetracycline, and erythromycin shown by human isolates were 96%, 96%, 29%, 57%, and 14%, while that shown by poultry isolates were 77%, 77%, 22%, 26%, and 17%, respectively. All quinolone-resistant strains contained a mutation in the *gyrA* gene ($T_{86} \rightarrow I_{86}$), suggesting that the strains were already widespread in Thailand.**

***Key words*: Antimicrobial drug resistance, *Campylobacter*, *gyr A* mutation**

Campylobacter jejuni is a major foodborne bacterium that affects children in many countries (4, 23, 35). In particular, this organism is isolated from the stool of diarrheal infants, animals, and contaminated food and water. Foods of animal origin (15, 25) or other cross-contaminated foods and drinking water (16) are considered to be the main source of this infection in humans. Previous reports have suggested that the prevalence of *C. jejuni* in children in Southeast Asian countries varies from 2.9% to 15% (27, 31, 37); in particular, 9.8% of Thai children with diarrhea have this infection (36). Poultry is considered to be the major reservoir of *C. jejuni*; however, in Thailand, the relationship between humans and chickens with regard to *C. jejuni* is poorly understood.

Another issue of concern with regard to *Campylobacter* is the increase in antimicrobial resistance that has appeared in various regions worldwide (3, 10, 26). Infection by these resistant *Campylobacter* may lead to suboptimal outcomes of antimicrobial treatment (34) or treatment failure (2). Antimicrobial resistance of both human and animal *Campylobacter* isolates has become increasingly common in Thailand and other developing countries (12, 26). Several studies in Thailand have identified a number of *Campylobacter* strains that are resistant to various antimicrobial agents, including quinolones (nalidixic acid) and fluoroquinolones (ciprofloxacin) (1, 26, 28).

It has been reported that a *C. jejuni* strain with high-level resistance to fluoroquinolone contains some point mutations in the Thr-86, Asp-90, and Ala-70 codons of the *gyr A* gene (5, 6, 38). A recent study has shown that a point mutation in the Thr-86 codon [wherein threonine (T_{86}) is replaced with isoleucine (I_{86})] in the *gyr A* gene directly leads to fluoroquinolone resistance in the *Campylobacter* spp. (7). Some earlier studies in Thai-

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Abbreviations: ABPC, ampicillin; Ala, alanine; Asp, aspartic acid; CPFX, ciprofloxacin; EM, erythromycin; I : Ile, isoleucine; mCCDA, modified charcoal-cefoperazone deoxycholate agar; MIC, minimum inhibitory concentration; NA, nalidixic acid; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; T : Thr, threonine; TC, tetracycline.

land found high proportions of *Campylobacter* resistant to fluoroquinolones (26, 27). However, in Thailand, convincing data on the *gyr A* gene mutation of *C. jejuni* isolates are unavailable.

The purpose of this study was to investigate the relationship between humans and chickens with regard to *C. jejuni* by using Penner's serotypes, random amplified polymorphic DNA (RAPD)-PCR analysis, and the antimicrobial resistance of the *C. jejuni* isolates obtained from humans and animals in Thailand. Furthermore, we conducted an experiment to determine the prevalence of fluoroquinolone-resistant *C. jejuni* in the isolates and to confirm the presence of a *gyr A* mutation in this organism.

Between 2001 and 2004, we collected 224 fecal and 15 blood samples from humans with diarrhea from several hospitals and 100 chicken rectal swabs from poultry farms in Thailand. Only the fecal samples of 209 patients were used, while both the blood and fecal samples of 15 patients were used. The human sample was obtained from patient prior to medical treatment. A single chicken rectal swab sample was collected from a building at a broiler production farm in which large numbers of birds were maintained in closed buildings. The chickens were administered coccidiostats (e.g., amprolium) and antimicrobial agents (including sulfamethoxazole and enrofloxacin) during the raising period but were not administered antimicrobial agents prior to sample collection.

Human feces (1 g), human blood (1 ml), or chicken rectal swab was added to 9 ml of Preston broth (Oxoid Ltd., Hampshire, England); this mixture was incubated at 42 C for 1 day under microaerophilic conditions comprising 5% O_2, 10% CO_2, and 85% N_2 by using a Gas pack jar system (Mitsubishi Chemical, Ltd., Tokyo). After incubation, the specimens were streaked onto modified charcoal-cefoperazone deoxycholate agar (mCCDA) plates (Oxoid) and incubated at 42 C for 3–5 days under microaerophilic conditions. A typical *Campylobacter* colony was selected from each specimen to further identify the strains by biochemical testing (curved, gram-negative strains showing positive catalase and oxidase test and the ability to hydrolyse hippurate). A total of 70 *C. jejuni* isolates from humans (69 isolates were from human fecal samples and one isolate was from a blood sample, and the organism was not detected in the feces of patients from whose blood samples *C. jejuni* was isolated) and 69 isolates from poultry were selected for serotyping by the Penner method with a commercially available serotyping kit (Denka, Tokyo) using the passive hemagglutination test (29), and analyzed for drug susceptibility by the agar dilution technique. Each antiserum included antibodies specific to the following antigenic components; A:1 and 44; B:2; C:3; D: 4, 13, 16, 43, and 50; E:5; F:6 and 7; G:8; I:10; J:11; K:12; L:15; N:18; O:19; P:21; R:23, 36 and 53; S:27; U:31; V:32; Y:37; Z:38; Z_2:41; Z_4:45; Z_5:52; Z_6:55; Z_7:57.

The antimicrobial susceptibility of the *C. jejuni* isolates was tested using the agar dilution technique, in accordance with the National Committee on Clinical Laboratory Standards (NCCLS) (19). The antimicrobial agents tested included nalidixic acid (NA), ciprofloxacin (CPFX), ampicillin (ABPC), tetracycline (TC), and erythromycin (EM). The concentrations of most antimicrobial agents tested in this study ranged from 0.25 to 256 mg/liter. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the antimicrobial agent that completely inhibited visible growth of the organism on the plates. The breakpoints for resistance to NA, CPFX, ABPC, TC, and EM were ≧32 mg/liter, ≧4 mg/liter, ≧32 mg/liter, ≧16 mg/liter, and ≧8 mg/liter, respectively. The breakpoints of the drugs were obtained from Human Isolates Final Report, 2003, the U.S. National Antimicrobial Resistance Monitoring System (NARMS), CDC (http://www.cdc.gov/narms/reports.htm) and were used to categorize *Campylobacter* into resistant groups.

We used the strains having the same Penner serotype and drug resistance profile. Using RAPD-PCR, we analyzed 8 isolates having serotype B and NA-CPFX-resistant *C. jejuni* of which 5 isolates were from humans and 3 from poultry, and 7 isolates having serotype C and NA-CPFX-TC-resistant *C. jejuni* of which 4 isolates were from humans and 3 from poultry. We used InstaGene matrix (BioRad, Hercules, Calif., U.S.A.) for extracting the DNA from *C. jejuni* strains and OPA-11 primer, 5'-CAATCGCCGT-3' for amplifying the DNA (11). The template DNA (1 µl) was subjected to PCR in a 25 µl (total volume) reaction mixture containing 5 µl of 5 × Flexi Buffer (Promega, Madison, Wis., U.S.A.), 3 µl of $MgCl_2$ (25 mM), 2.5 µl of dNTPs (1.25 mM of each dNTP), 0.2 µl of *Taq* polymerase (5 units/µl, Promega), 1 µl of OPA-11 primer (30 pmol), and 12.3 µl of DNase- and RNase-free distilled water. The program of amplification was as follows (24): 4 cycles of 94 C, 5 min; 36 C, 5 min; 72 C, 5 min and 30 cycles of 94 C, 1 min; 36 C, 1 min; 72 C, 2 min. After PCR amplification, electrophoresis was performed using 8 µl of the products on 3% agarose gel, followed by ethidium bromide staining and photography under UV light.

A part of the *gyr A* gene of 70 human and 69 poultry isolates used in this study was sequenced. We used newly designed primers for the amplification of the *gyr A* gene of the prototype *C. jejuni* strains (GenBank

Accession no. AL139077). Their sequences were 5'-CCT GAC GCA AGA GAT GGT TT-3' (Cjg-F) and 5'-CCA AAG TTG CCT TGT CCT GT-3' (Cjg-R) (theoretical amplicon, 227 nt). Template DNA (1 µl) was subjected to PCR in a 25 µl (total volume) reaction mixture containing 12.5 µl of PCR Master Mix (Promega), 10 pmol of Cjg-F and Cjg-R primers, and DNase- and RNase-free distilled water. The PCR protocol included incubation for 3 min at 94 C; this was followed by 30 cycles at 94 C for 1 min, 55 C for 1 min, and 72 C for 1 min, and an additional 5 min for elongation at 72 C after the last cycle. The sizes of the amplified DNA fragments were confirmed by electrophoresis using a 1.5% agarose gel. After the purification of the DNA fragments with the QIAquick PCR purification kit (Qiagen, Hilden, Germany), the nucleotide sequence was determined by using an automated DNA sequencer (ABI 310 DNA sequencer; Applied Biosystems, Foster City, Calif., U.S.A.) and a Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Prevalence of resistance or point mutation in the Thr-86 codon ware calculated by dividing the number of resistant or mutant isolates of *Campylobacter* by the total number of *Campylobacter* isolates processed. Testing for significant differences in resistant or mutant *C. jejuni* isolated from human and poultry samples was carried out by using the chi-square (χ^2) test.

C. jejuni was isolated from 31% of the human fecal samples (69/224), 1% of the human blood samples (1/15), and 69% of the chicken swab samples (69/100). As shown in Table 1, 36 of the 70 (51%) human isolates were classified into 10 serotypes. The most predominant serotype was B (9 strains), followed by the serotypes C (8 strains), R (5 strains), E (4 strains), G (3 strains), A (2 strains), K (2 strains), D (1 strain), I (1 strain), and L (1 strain). In the poultry isolates, 47 of the 69 (68%) isolates were classified into 9 serotypes, and the most predominant serotype was A (16 strains); this was followed by the serotypes C (7 strains), I (6 strains), K (6 strains), B (4 strains), E (4 strains), S (2 strains), D (1 strain), and L (1 strain). In this study, the serotyping of 34 (49%) of the 70 human strains and 22 (32%) of the 69 poultry strains could not be achieved by using a commercial serotyping kit. The prevalence of a particular serotype differs across countries based on their geographic location; for example, the most predominant serotypes in both humans and chickens in Japan are serotypes B, D, and L, while those in Denmark are serotypes B, A, and D (21, 22, 30, 33). In this study, we found that the serotypes B and C and the serotypes A and C were common in human and poultry *C. jejuni* isolates, respectively, in Thailand.

Table 1. Penner's serotype in *C. jejuni* isolates from humans and poultry in Thailand

Serotype	Human isolate	Poultry isolate
A	2	16
B	9	4
C	8	7
D	1	1
E	4	4
I	1	6
K	2	6
L	1	1
R	5	
S		2
G	3	
Untypable	34	22
Total	70	69

Table 2 shows the MIC distribution and resistance rates of *C. jejuni* isolated from humans and poultry in Thailand. The rate of resistance to NA, CPFX, ABPC, TC, and EM shown by human isolates were 96%, 96%, 29%, 57%, and 14% and that shown by the poultry isolates were 77%, 77%, 22%, 26%, and 17%, respectively. In general, a wide range of MICs of most antimicrobial agents was observed mainly among *C. jejuni* isolates from poultry than among those from humans. The MIC of CPFX for isolates from humans and poultry showed two peak characteristics, namely, low-dose MIC (0.025–1 mg/liter) and high-dose MIC (8–128 mg/liter). The MIC of TC for isolates from humans also showed two peak characteristics (low-dose MIC: 0.5–2 mg/liter; high-dose MIC: 16–64 mg/liter). The highest rate of antimicrobial drug resistance in the human and poultry isolates was to CPFX and NA (both 96%). Significant differences ($P < 0.01$) between the human and poultry isolates were observed with regard to resistance to NA, CPFX, and TC but not ABPC and EM. For the human strains, the rates of resistance to NA, CPFX, and TC were statistically higher than those of the poultry strains.

Table 3 shows the counts of *C. jejuni* with different resistance profiles. All human isolates (70/70 strains) possessed resistance to the agents used in this study and were classified into 7 profiles. The most predominant profile was that of NA-CPFX-TC (22 strains), followed by those of NA-CPFX (20 strains), NA-CPFX-ABPC-TC (10 strains), NA-CPFX-ABPC (5 strains), NA-CPFX-EM (5 strains), NA-CPFX-ABPC-TC-EM (5 strains), and TC only (3 strains). Among the poultry isolates, 11 strains were not found to be resistant to the agents. Of the 69 isolates, 58 (84%) were classified into 8 profiles, and the most predominant profile was that of NA-CPFX (26 strains); this was followed by