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RESEARCH ARTICLE

Occurrence of *Campylobacter* in Dairy and Beef Cattle and Their Farm Environment in Malaysia

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A B S T R A C T

Aim of the present study was to determine the occurrence of *Campylobacter* in cattle and their farms environment at two different incubation temperatures. A total of 248 samples including cattle (180), water (27), feed (7), cattle house floor (9), water trough (9) and flies (16) were collected from dairy and beef farms in Malaysia. All samples were cultured on *Campylobacter* blood free selective agar base (mCCDA- modified Charcoal Cefoperazone Deoxycholate Agar) and isolates were identified using multiplex PCR. Prevalence of *Campylobacter spp.* was 26.1% in cattle. *Campylobacter spp.* were isolated from 37, 33.3, 25, 14.3 and 11.1% of water, floor of the cattle houses, flies, feed and water trough samples, respectively. The overall isolation rates of *Campylobacter* was higher at 42ºC (25.4%) as compared to 37ºC (20.6%); however, the difference was not significant (P>0.05). It is concluded that *Campylobacter* are quite prevalent in cattle and farm environment which could be a potential source of human infection and environmental contamination.

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INTRODUCTION

Campylobacteriosis is one of the most common bacterial causes of human intestinal disease in both developed and developing countries (Scallan *et al*., 2011). In many European countries, prevalence of campylobacteriosis continues to increase and today it exceeds the number of salmonellosis cases (Silva *et al*., 2011). Most of the human foodborne diseases are caused by *Campylobacter jejuni* and *Campylobacter coli* (Uaboi-Egbenni *et al*., 2012). *Campylobacter* species colonize various species of wild and farm animals, principally poultry and birds, as part of their gut microbiota without causing infection.

In human populations, the incidence of *Campylobacter* infections is frequently associated with *Campylobacter* in poultry; however, cattle may also be a significant cause of human campylobacteriosis (Gilpin *et al*., 2008; Sanad *et al*., 2011). Human can be infected with *Campylobacter* from cattle by direct contact with infected animals, ingestion of raw milk or raw undercooked meat products and from contaminated environment (Heuvelink *et al*., 2007; Lynch *et al*., 2011; Kazemeini *et al*., 2011). A number of studies have compared the effect of different incubation temperatures on the isolation of *Campylobacter* species. Many laboratories use incubation temperature of 42ºC and it is best for isolation of thermophilic *Campylobacter* species. However, non-thermophilic *Campylobacter* species generally grow well at 37ºC (Engberg, 2006). The major source of human infection is raw or undercooked poultry meat but beef, pork, raw milk and water have also been associated with the infection. Although, there are many reports on incidence of *Campylobacter* in poultry and poultry products in Malaysia, little is known on occurrence of *Campylobacter* in cattle. Therefore, aims of present study were to isolate *Campylobacter* from dairy and beef cattle, and farm environment using two different incubation temperatures.

MATERIALS AND METHODS

Study Areas and samples collection: A total of 180 samples from cattle and 68 samples from farms environment from dairy and beef cattle farms were sampled at different locations in Selangor, Negeri

Sembilan and Malacca state. Rectal swab samples from cattle were collected using a sterile cotton swab and placed in a sterile bottle containing Cary-Blair medium (Oxoid). As for the water, samples of at least 100 ml each were taken from two different water troughs and a water tank. Five grams of feed, 5-10 flies, floor and water trough swab samples, were each placed in separate Bolton selective enrichment broth (Oxoid) supplemented with Bolton antibiotic supplements (Oxoid) and 5% lysed horse blood. All the samples were analyzed within 4-6 h after sampling.

Isolation and identification of bacteria: Rectal swab samples were directly streaked on *Campylobacter* blood free selective agar base (mCCDA- modified Charcoal Cefoperazone Deoxycholate Agar) (Oxoid) containing cefoperazone (16.0 mg) and amphotericin B (5.0 mg) supplement. A set of two plates were prepared for each sample and one set of plates were incubated at 42ºC and the other at 37ºC under microaerophilic condition for 48 h.

Each water sample was filtered through a sterile 0.45 µm pore-size cellulose acetate membrane filter (47 mm diameter) (Milipore, Sartorius Stedim, Germany). Each membrane filter was placed in a sterile bottle containing Bolton selective enrichment broth (Oxoid). For each environmental sample, a set of two bottles was prepared, one set was incubated at 42ºC and the other set at 37˚C in a microaerophilic atmosphere for 48 h. After incubation, each enriched mixture was then streaked onto mCCDA and incubated as mentioned above, depending on which bottles they came from.

Presumptive colonies on each mCCDA plate were then subcultured onto Columbia Blood Agar (CBA, Oxoid) with 5% defibrinated horse blood and incubated as described above, depending on which plates they came from. For identification, biochemical tests were carried out, namely: oxidase test, catalase test, indoxyl acetate hydrolysis test,hippurate hydrolysis test and urease test.

Multiplex PCR assay of *Campylobacter* species: The extraction of genomic DNA was performed using Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. PCR procedure was performed according to method of Yamazaki-Matsune *et al*. (2007). The sequences of the primers used for gene amplification are shown in Table 1. A total of 5 µl of template DNA was added to a mixture containing 2 µM of each primer and 25 µl of 2x Multiplex PCR Master Mix (Multiplex PCR Master Mix Kit, QIAGEN, USA). The final volume was adjusted to 50 µl with RNase free H_2O. DNA amplification was performed in Thermocycler (Eppendorf, Germany). The cycling conditions used were: one cycle of initial denaturation at 95ºC for 15 min, followed by 25 cycles of denaturation at 95ºC for 0.5 min, annealing at 58ºC for 1.5 min and extension at 72ºC for 1 min, and ending with a final extension time at 72ºC for 7 min. Each reaction mixture was analysed by gel electrophoresis through 3% (w/v) agarose in 1xTBE solution, and visualized by UV transillumination after staining with ethidium bromide.

Data analysis: In order to determine the isolation rate and strength of agreement between the two different incubation temperatures, Chi square test and strength of agreement using kappa test statistic were used. The prevalence among adults and calves and also among dairy and beef cattle were determined using Chi square test. The results were considered statistically significant at $P \leq 0.05$ on 95% CI using SPSS version 21.

RESULTS

Overall, 26.6% samples, including cattle rectal swabs and farm environment, were positive for *Campylobacter*. The prevalence of *Campylobacter* in dairy and beef cattle was 26.1% (Table 2). Six species of *Campylobacter* were identified by m-PCR assay (Fig. 1). The prevalence was

Table 1: Primer sequences used for the multiplex PCR assay and the predicted sizes of PCR products (Yamazaki-Matsune *et al.*, 2007)

Species	Size (bp)	Target gene	Primer	Sequence
Genus *Campylobacter*	816	16S rRNA	C412F	5′-GGATGACACTTTTCGGAGC-3′
			C1228R	5′-CATTGTAGCACGTGTGTC-3′
C. jejuni	735	hip gene	HIP400F	5′-GAA GAG GGT TTG GGT GGT G-3′
			HIP1134R	5′-AGC TAG CTT CGC ATA ATA ACT TG-3′
C.coli	894	ceuE gene	F	5′-ATG AAA AAA TAT TTA GTT TTT GCA-3′
			R	5′-ATT TTA TTA TTT GTA GCA GCG-3′
C. lari	251	glyA	CLF	5′-TAGAGAGATAGCAAAAGAGA-3′
			CLR	5′-TACACATAATAATCCCACCC-3′
C.fetus	359	cstA	MG3F	5′-GGTAGCCGCAGCTGCTAAGAT-3′
			CF359R	5′-AGCCAGTAACGCATATTATAGTAG-3′
C. hyointestinalis subsp. *hyointestinalis*	611	23S rRNA	HYO1F	5′-ATAATCTAGGTGAGAATCCTAG-3′
			HYOFET23SR	5′-GCTTCGCATAGCTAACAT-3′
C. upsaliensis	86	lpxA	CU61F	5′-CGATGATGTGCAAATTGAAGC-3′
			CU146R	5′-TTCTAGCCCCTTGCTTGATG-3′

Table 2: Species distribution of *Campylobacter* in cattle and their farm environment

Samples	Total number	No of positive (%)	*C. j* No(%)	*C. c* No(%)	*C. f* No(%)	*C.l* No(%)	*C.u* No(%)	*C. hyo* No(%)	OTC No(%)
Cattle	180	47*(26.1)	23(39.6)	5(8.6)	13(22.4)	3(5.2)	8(13.8)	4(6.9)	2(3.4)
Water	27	10*(37)	6(50.0)	-	-	-	6(50.0)	-	-
Flies	16	4(25)	1(25.0)	-	-	-	3(75.0)	-	-
Feed	7	1(14.3)	-	-	-	-	-	1(100)	-
House floor	9	3*(33.3)	-	-	-	1(25.0)	2(50.0)	-	1(25.0)
Water trough	9	1(11.1)	-	-	-	-	-	-	1(100)
Overall	248	66 (26.6)	30(37.5)	5(6.2)	13(16.2)	4(5.0)	19(23.7)	5(6.2)	4(5.0)

C. j: *C. jejuni*; *C. c*: *C. coli*; *C. f*: *C. fetus*; *C. l*: *C. lari*; *C. u*: *C. upsaliensis*; *C. hyo*: *C. hyointestinalis* subsp. *hyointestinalis*; OTC: unidentified isolates; *: colonization by two *Campylobacter* species.

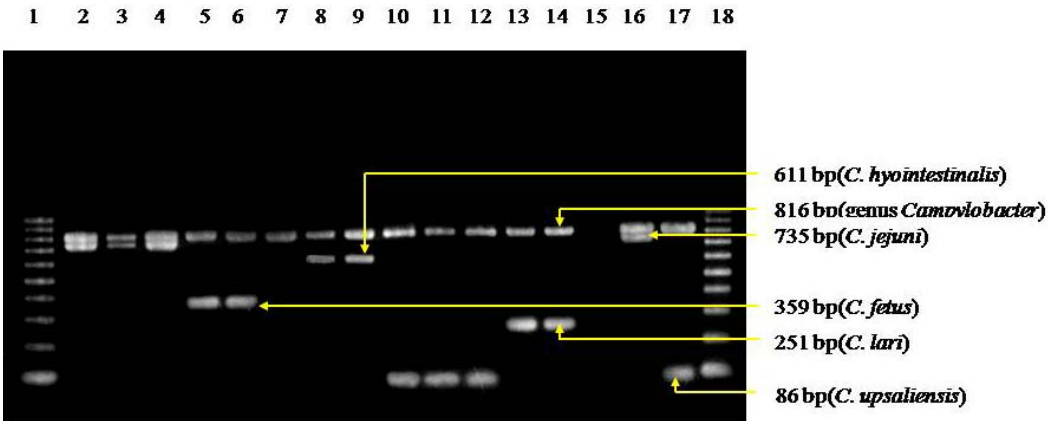

Fig. 1: m-PCR for *Campylobacter* species.
Lanes 1 and 18: 100 bp ladder; Lanes 2 to 4: C. *jejuni*; Lane 5 to 6: C. *fetus*; Lane 7: unidentified spp; Lane 8 to 9; C. *hyointestinalis*; Lane 10 to 12: C. *upsaliensis*; Lane 13: to 14: C. *lari*; Lane 15: negative control; Lane 16: C. *jejuni* CCUG 11284 A; Lane 17: C. *upsaliensis* CCUG 14913.

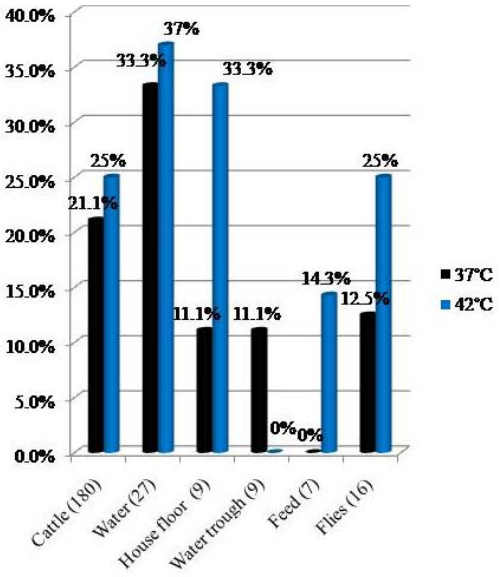

Fig. 2: Occurrence of *Campylobacter* isolated from cattle and farms environment when incubated in two different temperatures

higher in beef cattle (31.6%) compared to dairy cattle (23.6%) but the difference was not significant. The prevalence was significantly higher in calves (40%) than adult cattle (22.1%) ($P<0.05$). From the 68 environmental samples, namely water samples in water tank and water trough, flies, feed, house floor and water trough swabs, 27.9% samples were positive for *Campylobacter* (Table 2).

The overall isolation of *Campylobacter* when incubated under two different temperatures was higher at 42ºC (25.4%) when compared to 37ºC (20.6%) (Fig. 2); however, the difference was not statistically significant and kappa test statistic showed good agreement between the two different incubation temperatures ($0.6\leq k<0.8$).

DISCUSSION

Prevalence of *Campylobacter* was 26.1% in this study. Gilpin *et al.* (2008) reported that seasonal periodicity (spring and summer) may likely contribute to the higher prevalence of *Campylobacter* in dairy operations. Ellis-Iversen *et al.* (2009b) identified that management practices and farm characteristics might enhance the probability of *Campylobacter* shedding in the farms. The occurrence of *Campylobacter* in the farms were due to a number of factors, such as poor hygiene conditions of farm and farm environment, high population of flies and presence of wild birds in the farm area. *Campylobacter* was not detected in one of the farms was possibly due to the "clean" condition of the farm compared to the other farms and the collected environment samples from this farm were also negative for *Campylobacter*. The study observed that this farm practised good management and hygiene, such as use of treated water supply, use of vehicle dip, use of separate boots, footbath disinfection, manure cleaning frequency two times per day and control of pests and other animals.

Campylobacter jejuni (39.6%) was the most frequently isolated species in cattle in this study (Table 2). In most studies, *C. jejuni* was also reported as the most predominant species in most farms and slaughterhouses (Ellis-Iversen *et al*., 2009b; Grove-White *et al.*, 2010; Uaboi-Egbenni *et al*., 2012). *Campylobacter* was more prevalent among beef cattle (31.6%) than among dairy cattle (23.6%) but the difference was not significant. Wesley *et al.* (2000) suggested that different feed and higher animal density in beef cattle could favour the higher rate of *Campylobacter* spp. in beef cattle than dairy cattle. In this study, the higher prevalence of *Campylobacter* in beef cattle might be due to less hygienic condition of the beef farm and farm environment such as contaminated drinking water, feed, floor and water container and high population of flies. A significantly higher rate of *Campylobacter* infection was detected in calves (40.0%) than in adult cattle (22.1%). The finding is similar with previous studies which reported higher prevalence of *Campylobacter* in calves: 59% in adult and 75% in calves (Gilpin *et al*., 2008); 60.6% in adult and 86.5% in calves (Ramonaite *et al*., 2013). Gilpin *et al*. (2008) observed that young calves are born free of *Campylobacter* species; however calves can rapidly be infected by this organism from the infected cows and contaminated farm surroundings.

Broiler houses previously contaminated by *Campylobacter*, contaminated water and feed can act as a vehicle for horizontal spreading of the organism in the

farm (Silva *et al*., 2011). *Campylobacter* organisms have been isolated from ground water (Ugboma *et al*., 2013) and river water (Denis *et al*., 2011). Thus, water is a potential reservoir for transmission of environmental *Campylobacter*. In the present study, 37.0% water samples were *Campylobacter* positive. The sources of water in the farms were: tap water, well water, underground water, pond water and river water. In this study, *Campylobacter* was not detected in water samples in Farm 2, 4 and 7 mainly due to the water sources of these farms were tap water which was treated with chlorine. Wesley *et al*. (2000) also found that chlorinated drinking water was not a risk factor for *Campylobacter* and *Arcobacter* contamination in dairy herds. The contamination of *Campylobacter* spp. in water tank and water in water trough in this study may be due to contamination with infected faecal material and improper farm management which included accumulation of sediments in water trough. Ellis-Iversen *et al*. (2009a) suggested that improved water trough sanitation is a good management factor to prevent *Campylobacter* infection because standing water in unclean water troughs may be an important factor for constant reinfection among cattle. In addition, Wesley *et al*. (2000) supported this suggestion by using individual waterers which can help to reduce infection in cattle.

The pooled sample of cattle house floor swabs (33.3%) water trough swabs (11.1%) and feed sample (14.3%) were found contaminated with *Campylobacter*. The occurrence of *Campylobacter* in the farm environment may be a consequence of high stocking density and poor farm management. Four of the pooled samples of flies (25.0%) were found positive for *Campylobacter* species. *Campylobacter* was also identified in 5% of flies in chicken farms (Choo *et al*., 2011). These insects could be essential vector for the spread of *Campylobacter* to poultry, cattle and other animals. This study showed that flies could be vector for transmission of *Campylobacter* from contaminated environment to cattle in the farms or from infected animals to the environment. Similarly, *Campylobacter* contamination on the house floor could spread to other animals in the farms. The presence of *Campylobacter* species among cattle and their farm environment showed the significance of cattle as well as their surroundings as a potential source of human infection.

The study isolated 20.6% of *Campylobacter* when incubated at 37ºC and 25.4% at 42ºC incubation temperature but the difference was not significant. Six species of *Campylobacter* were isolated at both temperatures. From the isolation result, the study found that either of the incubation temperatures can be used for the isolation of *Campylobacter* species. However, incubation temperature 37ºC should be used for the isolation of non-thermophilic *Campylobacter* such as *C. fetus* which some may be missed when incubated at 42ºC.

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Author's contribution: SA, ZZ and MM designed, supervised and coordinated the research; WW conducted the study, data analysis and drafted the manuscript, while AA, GD and MA provided valuable input in laboratory work and data analysis. SA, ZZ, MM and WW interpreted the data, revised the manuscript, commented on and approved of the final version.

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