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Department of Veterinary Medicine and Public Health, Faculty of Veterinary Medicine, Sokoine University of Agriculture, Morogoro, Tanzania

Prevalence of Thermophilic *Campylobacter* Infections in Humans, Chickens and Crows in Morogoro, Tanzania

R. H. Mdegela[1,2], H. E. Nonga[1], H. A. Ngowi[1] and R. R. Kazwala[1]

Address of authors: [1]Department of Veterinary Medicine and Public Health, Sokoine University of Agriculture, PO Box 3021, Morogoro, Tanzania; [2]Corresponding author: Tel.: +25523 2604542; fax: 255232604647; E-mail: rmdegela@yahoo.com/ mdegela@suanet.ac.tz

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Summary

Prevalence of thermophilic *Campylobacter* infections in humans, chickens and crows was determined in a cross-sectional study that was carried out in urban and rural areas of Morogoro region, Tanzania during the period of January 2003 to December 2004. A total of 632 human stool samples, 536 cloacal swabs from local and broiler chickens and 22 intestinal contents from crows were screened for presence of thermophilic campylobacters using Skirrow's protocol. Representative *Campylobacter jejuni* isolates from human and chicken samples were also analysed by polymerase chain reaction (PCR) as a definitive identification method. The overall prevalence of thermophilic campylobacters was 9.3% (95% CI: 7.2–11.9), 69.8% (95% CI: 65.7–73.6) and 72.7% (95% CI: 49.8–89.3) in humans, chickens and crows respectively. In humans, 59 thermophilic campylobacters were isolated of which 96.6% were *C. jejuni* and 3.4% *Campylobacter coli*. There was a significantly ($P < 0.001$) higher prevalence in young individuals (16%) than in adults (7%). Of 341 isolates from chickens, 91.2% were *C. jejuni* and 8.8% were *C. coli*. A significantly ($P < 0.05$) higher infection rate was observed in rural local chicken (76%) than in broilers (60%). In crows, of 16 isolates, 93.8% were *C. jejuni* and 6.2% were *C. coli*. Definitive identification of *C. jejuni* by PCR revealed positive results in 74.1% of 243 analysed isolates. Findings in this study indicate high prevalence of thermophilic campylobacters in humans, chickens and crows in Morogoro, and a higher infection rate of *C. jejuni* than that of *C. coli* in different animal species. Age of humans and location of chickens were identified as risk factors for thermophilic *Campylobacter* infections. Positive isolates to biochemical tests that indicated negative results on PCR indicates the additional value of PCR for definitive diagnosis of *C. jejuni*.

Introduction

Campylobacteriosis, a food and waterborne zoonosis, is a disease of socio-economic significance worldwide (Dingle et al., 2000; Yolanda et al., 2002). Thermophilic campylobacters namely *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter lari* are the major causes of the disease. This pathogen has a considerable ecological diversity and can be isolated from food-producing animals, wild animals, birds and pet animals as normal flora as well as from the environment (On, 1996; Sandberg, 2002). Avian species, in particular poultry, are the most frequent warm-blooded animals colonized by thermophilic campylobacters (Yogasundram et al., 1989). Undercooked meat, particularly poultry meat, and contaminated drinking water contributes significantly to outbreaks of human campylobacteriosis worldwide (Koenraad et al., 1997; Jones, 2001; Diergaardt et al., 2004). Apart from poultry, other wild birds such as crows have also been shown to play a big role in the transmission cycle of *Campylobacter* to humans and other animals (Saleha, 2004). As crows feed on kitchen wastes and insects, they tend to pick campylobacters from potential sources and transmit to susceptible hosts (Jacobs-Reitsma et al., 1995).

Despite the limitation of information in African countries regarding human campylobacteriosis, there is evidence of its existence through studies that were conducted in Ethiopia, Kenya, Algeria, Cameroon, Zimbabwe, South Africa, Nigeria and Tanzania (Asrat et al., 1997; Jiwa et al., 1997; Coker et al., 2002). However, despite the substantial burden of the disease, generally the national surveillance programs for campylobacteriosis do not exist in most of the developing countries because much of the efforts are directed towards malaria, tuberculosis, cholera, trypanosomoses, onchocerciasis and schistosomiasis (Lambrechts et al., 1999; Anon., 2001).

The zoonotic nature of campylobacteriosis makes it important from clinical and economic perspectives worldwide (Coker et al., 2002). The economic losses due to *Campylobacter* infections are mainly related to treatment costs, loss of productivity for infected people and costs for controlling the pathogen (Skirrow and Blaser, 1992).

Campylobacteriosis may be diagnosed using conventional and molecular techniques. Bacterial culture is frequently used for routine isolation (Sandberg, 2002) and serology for serosurveillance of *Campylobacter* infections. Biochemical tests are used for phenotypic identification of campylobacters (Bolton et al., 1984). In Tanzania, limited studies have been conducted on *Campylobacter* infections in animals and humans (Kazwala et al., 1992a; Linblom et al., 1995; Jiwa et al., 1997). These studies established baseline data of *Campylobacter* infections in humans and animals using conventional isolation and identification methods. Although the conventional methods are useful, they have some limitations due to bacterial fastidiousness, asaccharolytic nature and possession of few distinguishing phenotypic characteristics

(Goossens and Butzler, 1992). Because of shortfalls associated with biochemical identification methods, several attempts have been made to use polymerase chain reaction (PCR) as a definitive identification method. Although definitive identification of thermophilic campylobacters by molecular techniques is relatively easy and rapid approach with high discriminatory power (Waegel and Nachamkin, 1996), such methods have not yet been applied in Tanzania. In addition, studies of thermophilic *Campylobacter* infections in non-conventional animals such as crow are limited in Tanzania. Thus the aim of this study was to determine the prevalence of thermophilic campylobacters in humans, poultry and crows in Morogoro urban and peri-urban areas using conventional isolation and identification methods and establish a PCR method for definitive identification of the pathogen. Information accrued through this study would improve the diagnostic capacity of the pathogen and provide additional epidemiological data for thermophilic campylobacters in Tanzania.

Materials and Methods

Study areas and source of samples

This study was conducted in Morogoro Municipality and Mkuyuni, Changa and Kibwaya villages located in Morogoro Rural district. Study villages were selected conveniently based on accessibility and timely shipment of samples to Sokoine University of Agriculture (SUA), where samples were analysed. Morogoro regional hospital, SUA dispensary and Upendo laboratory in Morogoro Municipality as well as Mkuyuni health centre served as sources of human samples. In Morogoro Municipality, chicken samples were collected from broilers and local chickens, while in the study villages samples were collected from local chickens only. The Indian and White necked crows which were also sampled during this study were trapped from different parts of Morogoro Municipality.

Sample collection, and isolation and identification of thermophilic campylobacters

Medical laboratory technicians collected human stool samples from patients who attended health facilities during sampling days and presented enteric signs that included at least diarrhoea, stomach cramps, nausea, vomiting and fever. Age, sex and place of residence for patients submitting samples were recorded. Patients below 15 years old were recorded as young and 15 years and above as adult. Medical laboratory technicians asked the patients or parents in case of children to collect stool samples using sticks of match-boxes. Collected samples were handled to laboratory technicians who immediately placed samples in universal bottles containing nutrient broth with Preston supplements, and then stored at 4°C before being shipped to laboratories for analysis within 8 h post-sampling. In the laboratory, the universal bottles with human stool samples enriched in nutrient broth with Preston supplements were incubated at 37°C for 24 h. After 24 h of incubation, the universal bottles with enriched samples were properly shaken and by using a sterile wire loop, a loopful of enrichment culture was subcultured onto modified charcoal, cefoperazone, deoxycholate, agar (mCCDA) (Oxoid Ltd, Basingstoke, UK) for primary isolation of thermophilic campylobacters.

Moistened sterile cotton wool swabs were used to collect the cloaca contents from chickens. Swabs with samples were immediately placed in sterile universal bottles containing 10 ml of freshly prepared Cary Blair broth. Universal bottles with samples were immediately placed in a cool box and transported under ice to the University laboratory. Each universal bottle with a cloacal swab was opened aseptically and the cloacal swabs were streaked onto mCCDA for primary isolation of thermophilic campylobacters.

Crows for sampling were examined clinically then sacrificed, opened the abdominal cavity and the gastrointestinal tract (GIT) was removed and put on a clean glass plate. Determination of sex was performed after opening the abdominal cavity. The GIT was straightened in order to expose the caecum. Using a sterile pair of scissors, a small cut was made through the caecal wall to expose caecal contents. Using a sterile wire loop, little caecal contents were streaked onto mCCDA for primary isolation of thermophilic campylobacters.

After direct inoculation of chicken and crow samples or the pre-enriched stool, inoculated Petri dishes were loaded in microaerophilic candle jars (Coldstream Engineering Ltd, Arista, Sweden) with a lighting candle and closed tightly. The microaerophilic candle jars with Petri dishes were incubated at 43°C for 48 h as described by Skirrow and Benjamin (1980). Suspected colonies were purified by subculturing on blood agar (Oxoid Ltd) and re-incubated at 43°C under microaerophilic environment for 24 h. Suspected *Campylobacter* colonies on blood agar were examined by Gram-staining and motility test. Bacteria that were Gram-negative curved or spiral rods and showed corkscrew like motion were subjected to further biochemical tests. Biochemical tests included catalase, oxidase, nitrate reduction, hippurate hydrolysis, growth at 43°C and their susceptibility to 30 μg cephalothin disks. These tests were carried out as described by Skirrow and Benjamin (1980) and Vandamme and Goossens (1992).

Typing of *C. jejuni* by PCR

About 60% of 398 *C. jejuni* isolates from humans and chickens that were confirmed phenotypically were randomly selected and tested by PCR (Nachamkin et al., 1993). Briefly, the isolates of *C. jejuni* from the stored stock were grown at 43°C overnight on blood agar under microaerophilic environment. A loopful of bacterial colonies from a fresh culture was washed with 1 ml of sterile double distilled water and resuspended in 200 μl of distilled water in an Eppendorf tube. The suspension was heated at 100°C for 30 min in a boiling water bath, then placed on ice for 5 min, and then centrifuged for 2 min in a microcentrifuge at 13 000 ***g*** to extract DNA. Five microlitres of the supernatant was then used for the PCR procedures. The PCR amplification was performed in a final volume of 50 μl containing 1X reaction buffer (50 mM KCl, 10 mM Tris–HCl; pH 9.0 at 25°C and 0.1 Triton ×100; Promega, Madison, WI, USA), 3.0 mM magnesium chloride, 200 μM of each deoxynucleoside triphosphates, 2.5 U of Taq DNA polymerase (Promega) and 50 pmol of each of the primers (Integrated DNA Technologies, Cape Town, South Africa). The forward primer sequence used was 5′-GGA TTT CGT ATT AAC ACA AAT GGT GC-3′, corresponding to nucleotides 1–26 in *flaA* gene, and the reverse primer 5′-CTG TAG TAA TCT TAA

AAC ATT TTG-3′ corresponding to nucleotides 1705–1728 of *flaA* on bases of previous sequence data (Fischer and Nachamkin., 1991). The reaction mixture was then overlaid with 50 μl of mineral oil (Sigma Chemical Co. Reagent, St Louis, MO, USA) to prevent evaporation.

Five microlitres of template DNA was denatured by incubation in boiling water for 10 min. This was followed by immediate quenching on ice before adding 5 μl to the 45 μl of the master-mix in a 0.5 ml Eppendorf tube to make a final volume of 50 μl of the reaction mixture. The DNA amplification was carried out by using an automated thermolcycler machine (Crocodile II Appligene Inc., Pleasanton, CA, USA). The amplification process started with denaturation at 94°C for 5 min, then 35 cycles at 92°C for 30 s followed by annealing at 55°C for one-and-half minutes and extension at 72°C for two-and-half minutes, and finally incubation at 72°C for 5 min. The PCR products were then maintained at 4°C until analysis. After amplification, 10 μl of the amplicon was analysed on 0.8% agarose gel. The presence of 1720-bp band indicated a positive result for *C. jejuni* isolate.

Data analysis

Data were analysed using Epi Info version 6 statistical software (Coulombier et al., 2001). Using statcalc, proportions of categorical variables were computed and further compared using chi-square test at critical probability of $P < 0.05$. The strength of associations between dependent and independent variables were determined using 2×2 contingency tables.

Ethical considerations

The permission to carry out this study was granted by the Tanzania National Institute for Medical Research before the human sampling started. Confidentiality of the study participants was strictly observed and after processing of samples, results were given to the medical personnel at respective health facility for further follow up of the patient.

Results

The overall prevalence of thermophilic campylobacters was 9.3% (95% CI: 7.2–11.9, $n = 632$), 69.8% (95% CI: 65.7–73.6, $n = 536$) and 72.8% (95% CI: 49.8–89.3, $n = 22$) in humans, chickens and crows respectively (Fig. 1). In humans, 59 thermophilic campylobacters were isolated of which 96.6% were *C. jejuni* and 3.4% were *C. coli* (Table 1). A highly significant ($P < 0.001$) infection rate was found in young 15.4% ($n = 175$) than in adults 7% ($n = 457$, RR = 2.34, 95% CI = 1.44–4.63). Sex and location of patients were not risk factors for thermophilic *Campylobacter* infections. *Campylobacter jejuni* was a predominant species of thermophilic campylobacters in all categories of patients and *C. coli* was only isolated in one young male patient from Morogoro municipality (Table 1).

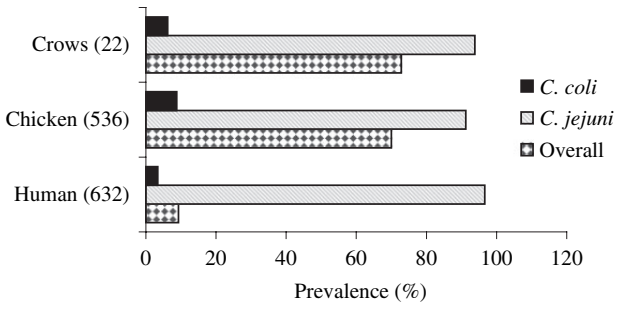

Fig.1. Prevalence of thermophilic campylobacters in different sources in Morogoro urban and rural areas (number in parentheses represent total number of samples examined).

Whereas 91.2% of 341 thermophilic campylobacters isolated in chickens were *C. jejuni*, 8.8% were *C. coli*. Infection rate in local chickens was significantly higher in chickens sampled in rural areas than in urban areas ($P < 0.01$, RR = 1.3, 95% CI = 1.25–3.51). Category of the chicken, type of management and flock size were found not to be risk factors for thermophilic *Campylobacter* infection in chickens (Table 1). In broilers, the infection rate of thermophilic campylobacters increased with age (Fig. 2). Like in humans, there were higher isolation rate of *C. jejuni* (91.2%) than that of *C. coli* (8.8%) in chickens. In crows of 16 isolates, 93.8% were *C. jejuni* and 6.2% were *C. coli*. Type and sex of crows were not risk factors for thermophilic *Campylobacter* infections.

Identification of 243 *C. jejuni* isolates by PCR revealed 74.1% positive amplification results after a simple water extraction of the chromosomal DNA. The results showed that 24.5% ($n = 49$) and 26.3% ($n = 194$) of the 243 human and chicken isolates, respectively, tested negative on PCR (Fig. 3).

Discussion

This study has demonstrated a high prevalence of thermophilic campylobacters in humans, chickens and crows in study areas. Such a high isolation rate of thermophilic campylobacters in humans, chickens and crows has also been reported in other studies (Saleha et al., 1998; Magistrado et al., 2001; Sandberg, 2002; Ringoir and Korolik, 2003; Saleha, 2004).

The established high prevalence of the thermophilic campylobacters of 9.3% in humans in Morogoro is significant especially in this era of HIV/AIDS. Elsewhere the incidence of campylobacteriosis in patients with HIV/AIDS has been reported to be 39 times higher than the infection rate in the general population (Altekruse et al. (1999). Since, HIV/AIDS cases have been predicted to double by the year 2020, unless serious control and prevention measures are taken, it is likely that *Campylobacter* cases in the sub-Saharan Africa will also increase (Coker et al., 2002). It is thus imperative that thermophilic campylobacters be considered in a different perspective because of its close association with HIV/AIDS which is a new pandemic and of economic significance in developing countries.

In this study, the prevalence of thermophilic campylobacters was significantly higher in young individuals than in adults, which is a similar finding to other studies (Blaser, 1997). Factors that are likely to contribute to high infection rates in young individuals in the study area are poor hygiene and sanitation, closeness to animals, malnutrition and low immunity because of first exposures. The health status and age of the host and *C. jejuni*-specific humoral immunity from previous exposure influence clinical outcome after infection (Calva et al., 1988; Altekruse et al., 1999). Furthermore, young individuals are more frequently taken to attend health facilities than adults and therefore higher possibility for thermophilic

Table 1. Prevalence and risk factors for thermophilic campylobacters infections in humans, chickens and crows

Risk factors	Category	Sample (*n*)	Prevalence (%)	RR	95% CI	Isolates (*n*)	*Campylobacter jejuni* (%)	*Campylobacter coli* (%)
Humans								
Age	Young	175	15.4	2.34	1.44–4.63*	27	96.3	3.7
	Adult	457	7.0			32	96.9	3.1
Sex (adults)	Male	216	7.4	1.04	0.51–2.47	16	100	0
	Female	241	7.1			16	93.8	6.3
Location	Rural	134	10.4	1.22	0.53–2.27	14	100	0
	Urban	498	9.0			45	95.6	4.4
Chicken								
Type	Local	335	71	1.1	0.73–1.62	236	86	14
	Broilers	201	69			138	100	0
Management	Extensive	325	72	1.1	0.85–1.86	233	85	14.2
	Intensive	211	67			141	100	0
Location (local only)	Rural	223	76	1.3	1.25–3.51*	169	87.6	12.4
	Urban	112	60			67	82.1	17.9
Crows								
Type	White necked	4	100	1.5	1.08–2.08	4	100	0
	Indian	18	67			12	92	8
Sex	Female	14	71	1.05	0.62–1.77	10	100	0
	Male	8	75			6	83	17

*Significantly different $P < 0.05$.

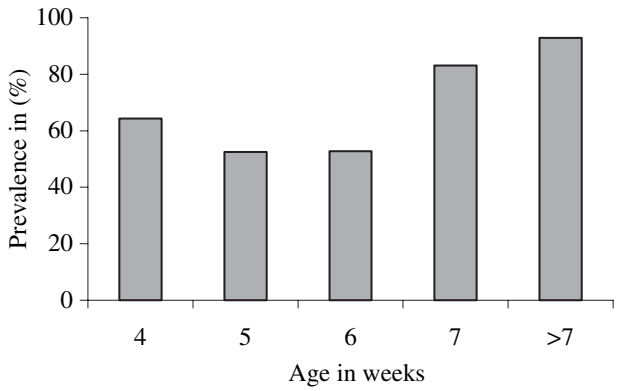

Fig. 2. Prevalence of thermophilic campylobacters in different age of broilers in Morogoro municipality.

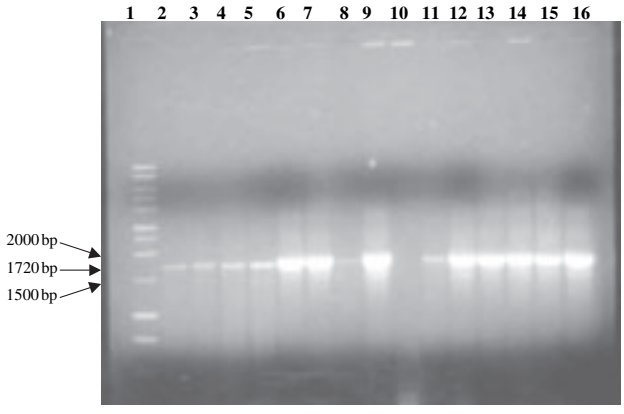

Fig. 3. Agarose gel (0.8%) showing amplification products of *Campylobacter jejuni* from humans and chickens. The desired product is at 1720. Lane 1 is a molecular weight marker (1 kb ladder), lanes 2, 3, 4, 5, 6, 7, 8 and 9 are chicken *C. jejuni* positive amplicons and lane 10 is a negative human *C. jejuni* amplicon while lanes 11, 12, 13, 14, 15 and 16 are positive human *C. jejuni* amplicons.

Campylobacter isolation rates in young than in adult individuals. Findings of the present study further showed that people screened in rural areas had comparable *Campylobacter* infection rate to those in urban areas ($P > 0.05$) despite their differences in life style and living standards. Similar prevalence in urban and rural areas suggests an existence of similar environmental factors and human behaviours that enhance survival and transmission of the pathogen.

The overall prevalence of thermophilic campylobacters in chickens was very high and there was no significant difference ($P > 0.05$) between local chickens (71%) and broilers (69%). The local chickens that were under extensive type of management might have been exposed to thermophilic campylobacters through carrier insects, vermin, rodents, kitchen wastes, contaminated water and environments, and possibly other infected animal species that increased the chances of infections (Adekeye et al., 1989; Saleha et al., 1998). On the other hand, poor housing hygiene and intensive management enhances transmission of campylobacters among broilers. Because of the existence of many risk factors for transmission of the pathogen in broilers and in local chickens, the prevalence of thermophilic campylobacters in the two categories of chickens was comparable, a finding that was similar to previous studies (Adekeye et al., 1989; Saleha et al., 1996).

Local chickens in rural areas were significantly more infected with thermophilic campylobacters than those in urban areas ($P < 0.01$). This might be due to treatment and other disease control measures, which are commonly practised, in local chickens kept in urban areas.

Although the minimum age of birds was 4 weeks, the isolation rate of *Campylobacter* spp. in broiler chicken was found to increase with age, a finding that was similar to the previous observations (Kazwala et al., 1992b). Chicks can become colonized with *Campylobacter* within 1–7 days of hatching and the bacterial burden may reach up to 1.2×10^7 CFU/g as the age increases (Saleha et al., 1998). In the present study, *Campylobacter* infection rate in chickens increased with age. In 4-week-old chicks, the infection rate was

64.3%, but significantly increased up to 93% in broilers by the age of 39 weeks. This was attributed to prolonged exposure to *Campylobacter* spp.

The ecology of *C. jejuni* involves wildlife reservoirs, particularly wild birds because of chances of contaminating water sources, environment and food and in turn transmitting the pathogens to humans and poultry (Luechtefeld et al., 1982). Rosef (1981) investigated the occurrence of *C. jejuni* in crows and reported a prevalence of 24.8%. Ito et al. (1989) isolated *C. jejuni* from several species of wild birds including crows. In addition, Saleha (2004) established a prevalence of 26% of *C. jejuni* in free flying crows around chicken flocks in Malaysia. Further studies in Nigeria showed phenotypical and genotypical similarities among *C. jejuni* isolates from free flying birds and humans (Adegbola et al., 1990). Thus the high isolation rate of thermophilic campylobacters of 72.8% established in this study and reported for the first time in Tanzania is of epidemiological significance. Free flying birds including crows around poultry farms may transmit thermophilic campylobacters to chickens if they get access to the rearing houses (Genigeorgis et al., 1986; Altekruse et al., 1999). Although, the isolates were not genetically characterized to the level of linking up the infections, it is possible that crows are among sources of thermophilic campylobacters in humans and chickens in Tanzania.

The predominant *Campylobacter* species in humans, chickens and crows was *C. jejuni*, which is the main aetiology of *Campylobacter* enteritis in man. This indicates that *Campylobacter* infection may be one of the major causes of enteritis of man in Tanzania (Linblom et al., 1995; Jiwa et al., 1997). These findings are in agreement with the report by Saleha et al. (1998), Altekruse et al. (1999) and Friedman *et al.* (2004) who observed a significant higher infection rate of *C. jejuni* than *C. coli* in human samples. Findings from this study, which are similar to findings in previous studies (Linblom et al., 1995; Jiwa et al., 1997; Sandberg, 2002); suggest that *C. coli* is not a common pathogen of campylobacteriosis in different species studied. The significant high isolation rate of *C. jejuni* from chickens and crows observed in this study signifies the role of such birds as carriers for the infection to man.

Findings from the present study have shown that of all 243 *C. jejuni* isolates confirmed positive by biochemical tests, 25.9% were negative on molecular identification by PCR that confirms the low discriminatory power of conventional biochemical tests when compared with DNA-based techniques. *Campylobacter* is a taxonomically complex genus (On, 2001), and *C. jejuni* strains have a wide genetic diversity (Newell et al., 2000; On, 2001). *Campylobacter jejuni* comprises two genetically distinct but highly related subspecies, *C. jejuni* ssp. *jejuni* and *C. jejuni* ssp. *doyley* both of which test positive on hippurate hydrolysis. As the latter has no known animal reservoir and is infrequently observed in human disease, most attention is frequently focused on *C. jejuni* ssp. *jejuni*. Thus most of the PCR tests have been developed for *C. jejuni* ssp. *jejuni* strains (On and Jordan, 2003) including the PCR method that was used in the present study (Nachamkin et al., 1993). The inability of many *C. jejuni* PCR tests to recognize strains of the latter taxon suggest that the genetic difference between the two subspecies may contribute to PCR negative results (On and Harrington, 2000). Therefore, it is possible that the *C. jejuni* that indicated negative results on PCR despite being positive on biochemical tests were of the subspecies *doyley*. Moreover, Diergaardt et al. (2004) in their study in South Africa found that culture isolates on mCCDA that were morphologically and biochemically similar to *C. jejuni* were found to be *Arcobacter* on subsequent testing using their partial 16S rDNA gene sequence as a molecular identification method. Based on these drawbacks of culture techniques and biochemical identification methods, it is possible that the 25.9% *C. jejuni* negative isolates on PCR might not be real *C. jejuni* which demonstrates the importance of applying a wide array of tests for definitive identification of *C. jejuni*.

In conclusion, findings from this study indicate a high prevalence of thermophilic campylobacters in particular *C. jejuni* in humans, chickens and crows. Given the drawbacks of conventional isolation and identification methods for campylobacters, it is recommended that a range of biochemical reactions should be used for preliminary identification of suspect isolates of *C. jejuni* and further definitive confirmation by PCR method.

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