APMIS ACTA PATHOLOGICA, MICROBIOLOGICA ET IMMUNOLOGICA SCANDINAVICA

APMIS 124: 508–515
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DOI 10.1111/apm.12531

Detection of *Campylobacter* in human and animal field samples in Cambodia

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Osbjer K., Tano E., Chhayheng L., Mac-Kwashie A.O., Fernström L.-L., Ellström P., Sokerya S., Sokheng C., Mom V., Chheng K, San S., Davun H., Boqvist S., Rautelin H., Magnusson U. Detection of *Campylobacter* in human and animal field samples in Cambodia. APMIS 2016; 124: 508–515.

Campylobacter are zoonotic bacteria and a leading cause of human gastroenteritis worldwide with *Campylobacter jejuni* and *C. coli* being the most commonly detected species. The aim of this study was to detect *Campylobacter* in humans and livestock (chickens, ducks, pigs, cattle, water buffalo, quail, pigeons and geese) in rural households by routine culturing and multiplex PCR in faecal samples frozen before analysis. Of 681 human samples, 82 (12%) tested positive by PCR (*C. jejuni* in 66 samples and *C. coli* in 16), but none by routine culture. Children were more commonly *Campylobacter* positive (19%) than adult males (8%) and females (7%). Of 853 livestock samples, 106 (12%) tested positive by routine culture and 352 (41%) by PCR. *Campylobacter jejuni* was more frequent in chickens and ducks and *C. coli* in pigs. In conclusion, *Campylobacter* proved to be highly prevalent by PCR in children (19%), ducks (24%), chickens (56%) and pigs (72%). Routine culturing was insufficiently sensitive in detecting *Campylobacter* in field samples frozen before analysis. These findings suggest that PCR should be the preferred diagnostic method for detection of *Campylobacter* in humans and livestock where timely culture is not feasible.

Key words: *Campylobacter*; Cambodia; humans; livestock; zoonosis.

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Campylobacter are zoonotic bacteria and a leading cause of human gastroenteritis worldwide (1), with symptoms ranging from diarrhoea, abdominal pain and fever to late sequelae such as reactive arthritis and, although rarely occurring, neurological Guillain–Barré syndrome (2). The vast majority of *Campylobacter* infections diagnosed in humans are caused by *Campylobacter jejuni* and *C. coli* (3).

In animals, on the other hand, *Campylobacter* commonly colonize the intestinal tract without causing any symptoms, with the exception of *C. fetus* causing reproductive failure (3). Transmission of *Campylobacter* to humans occurs through ingestion of contaminated food or water, or through direct contact with animals, particularly poultry (4).

The epidemiology of human campylobacteriosis appears to differ between high- and low-income countries (5). In high-income countries symptomatic infection occurs in all age groups (2), whereas in low-income countries most symptomatic *Campylobacter* infections are diagnosed in young children and infection in adults is believed to be asymptomatic (5). Nevertheless, both symptomatic and asymptomatic infections have been shown to

Received 10 November 2015. Accepted 7 February 2016

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be associated with undernutrition and stunting (6, 7). The best-known sources of human campylobacteriosis in high-income countries are consumption of chicken meat, unpasteurized dairy products and contaminated water, and direct contact with farm animals (8, 9). Data on source attribution in low-income countries are scarce, but sources include poor hygiene conditions, keeping animals in the house, manure and wet litter in house yards and drinking contaminated water (10–12). *Campylobacter* are prevalent in humans and livestock throughout Southeast Asia and are reported to be the most commonly identified bacterial pathogens in children with diarrhoea in Vietnam and Thailand (13, 14). In a recent study on livestock in Vietnam, *Campylobacter* were detected by culture in 32% of poultry and 54% of pigs sampled in low-biosecurity farms (15), whereas in Cambodia *Campylobacter* were detected by culture in 81% of the poultry carcases available on sale in markets (16).

Data on enteropathogens in the Cambodian population is limited. To the best of our knowledge, there are no publications on the prevalence of *Campylobacter* in humans studied outside hospital facilities. Likewise, there are no studies available on the prevalence and distribution of *Campylobacter* among livestock on Cambodian farms. Faecal culture, targeting viable bacteria, is the traditional technique used for detection of *Campylobacter*. In screening of livestock samples, enrichment is routinely practiced prior to plating, to enable detection of low numbers of *Campylobacter*. In human samples, however, culturing without enrichment is routinely used and common in Cambodia for diagnosis in diarrhoea patients, although enrichment is ocassionally used (17). In contrast to many other gastrointestinal pathogens, *Campylobacter* are more difficult to culture due to their sensitivity to oxygen and to changes in temperature (18). Sample transport at low temperature and rapid culture are key factors for successful detection, but can be difficult to achieve under field conditions when diagnostic facilities are far away. In such conditions, freezing of samples and PCR assay could serve as an alternative method. PCR has been shown to have higher sensitivity than *Campylobacter* culture, as it detects Campylobacter DNA and not only live bacterial cells (19, 20). The aim of this study was to analyse faecal samples, collected concurrently from humans and livestock in rural Cambodian households, for the presence of *Campylobacter* and to assess the performance of routine culturing and multiplex PCR in samples collected under rural field conditions and frozen before being analysed.

MATERIALS AND METHODS

Study design and sample collection

This cross-sectional study involved three Cambodian regions: Kampong Cham province in the central lowlands, Battambang province in the north-west wetlands and Kampot province on the south coast (Fig. 1). Samples were collected during 10 days per region, with collection in Kampong Cham province in May 2011, in Battambang province in July 2012 and in Kampot province in March 2013. In each region, 10 villages were included and in each village, 7–10 households were sampled. Details about the purposive selection of villages and households are described elsewhere (21). Each village was visited for two consecutive days. On day 1, households selected for participation were informed about the study, asked for their consent and provided with plastic containers for human faecal samples. All members of the household were encouraged to provide a faecal sample, regardless of gender, age and history of gastrointestinal symptoms. On day 2, all human samples produced were collected and one to six livestock, including chickens, ducks, pigs, cattle, water buffalo, quail, pigeons and geese, were sampled from the same households. Geographical position at the centre of the villages included in the study was recorded using a handheld global positioning system (Garmin eTrex H).

The human faecal samples, self-collected by participants, were stored on ice-packs in cooler bags for a maximum of 6 h until faecal material was transferred with sterile cotton swabs into vials by the survey team. Poultry and bird samples were collected by insertion of a sterile cotton swab into the cloaca, whereas buffalo, cattle and pig samples were collected by dipping a sterile cotton swab into faecal material collected manually from the rectum. One swab was collected from each individual livestock, while duplicate swabs were taken from human samples. The swabs with human and livestock faecal material were placed in 2-mL vials containing 1 mL bacterial freeze medium, namely Luria broth supplemented with 20% glycerol (Uppsala University, Uppsala, Sweden). The vials were stored in cooler boxes or refrigerators for a maximum of 20 h and then transported on ice to Phnom Penh, which took 3–8 h. In Phnom Penh, human samples were sent to the laboratory at the National Institute of Public Health (NIPH) and livestock samples to the laboratory at the National Veterinary Research Institute (NaVRI) for storage at −70 °C for 1–12 months. One of the duplicate human samples and all livestock samples were then shipped in plastic boxes containing dry ice to Uppsala University (UU) and the Swedish University of Agricultural Sciences (SLU) respectively. In Sweden, all samples were stored at −70 °C for a maximum of 6 months prior to analysis.

Ethical approval (no. 43, 8 April 2011) was obtained prior to the study from the National Ethics Committee for Health Research, Ministry of Health, Cambodia, and an advisory ethical statement (Dnr 2011/63) was obtained from the Regional Board for Research Ethics in Uppsala, Sweden.

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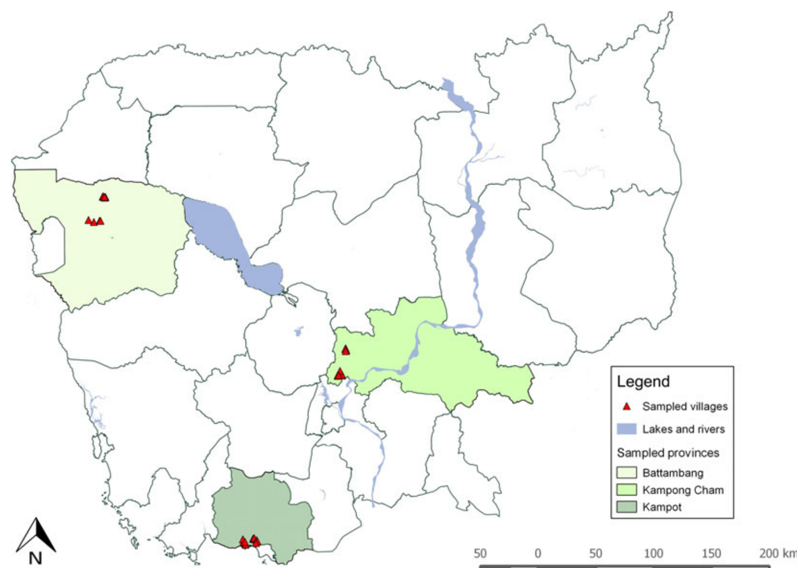

Fig. 1. Map of Cambodia showing the geographical distribution of the 30 villages included in the cross-sectional study in 2011–2013.

Laboratory analyses

Culture of human samples

A total of 681 human faecal samples originating from 269 households were cultured at NIPH in Cambodia. In addition, 200 randomly selected duplicate samples were cultured at the Clinical Microbiology Laboratory at UU. A diagram of the analytical procedure used for detection of *Campylobacter* in faecal samples from humans is shown in Fig. 2A. Samples were thawed and gently vortexed before faecal material was spread on agar plates. Culturing at NIPH was performed on Campylobacter agar base with antimicrobic supplement Blaser and 10% sheep's blood (Becton, Dickinson and Company, Sparks, MD, USA). The plates were incubated in a microaerobic atmosphere (GasPak EZ Gas Generating Pouch Systems; Becton, Dickinson and Company) at 42 °C for 48 h. *Campylobacter jejuni* type strain (ATCC 81146) and *C. coli* type strain (ATCC 43474) were used as controls. Culture at UU was performed on modified charcoal–cefoperazone–deoxycholate agar plates (mCCDA) (Oxoid, Basingstoke, UK). The plates were incubated in a microaerobic atmosphere (CampyGen; Oxoid) at 42 °C for 48 h. The type strain *C. jejuni* (81–176) was used as a control. Identification of *Campylobacter* was based on typical microscopical appearance (curviform bacteria exhibiting spin movement) and positive reactions to catalase and oxidase.

Culture of animal samples

A total of 853 livestock samples originating from 249 households were cultured at SLU using ISO 10272:2006 with type strains *C. jejuni* (SLV 267) and *C. coli* (SLV 427) as controls. A diagram of the analytical procedure used for detection of *Campylobacter* spp. in faecal samples from livestock is shown in Fig. 2B. Enrichment and incubation steps were performed in a microaerobic atmosphere (CampyGen; Oxoid). Samples were thawed and each cotton swab transferred to a sterile screwcap vial containing 10 mL Bolton broth (Oxoid) with antibiotic supplement SR0183 (Oxoid), 1% ethanol and 5% lysed horse blood (Håtunalab AB, Håtuna, Sweden). Enrichment was carried out at 37 °C for 48 h, after which two loops of surface material were spread on mCCDA (Oxoid) and cultured at 41.5 °C for 48 h. Up to five suspected *Campylobacter* colonies were picked from each mCCDA plate and subcultured onto blood agar plates (National Veterinary Institute, Uppsala, Sweden) at 41.5 °C for 24 h. Isolates with curviform bacteria exhibiting spin movement and positive reactions to catalase and oxidase were identified as *Campylobacter* spp. Identification to species level was based on hippurate hydrolysis. Positive isolates were identified as *C. jejuni* and negative isolates were further analysed by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry (22).

Molecular detection by PCR in human and animal samples

DNA was extracted from the human samples using the easyMAG NucliSENS extractor Off-board protocol (bioMérieux SA, Marcy l'Etoile, France), extracting 500 μL faecal suspension. DNA was extracted from the livestock samples using QIAmp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) following a modified protocol, where the extracted volume was increased to 400 μL faecal suspension. Samples were kept at −20 °C until the multiplex PCR was performed. Identical multiplex PCR was performed on DNA extracted from human and livestock samples using primers specific for *C. jejuni* and *C. coli*. For *C. jejuni*, the primer pair MDmapA1 upper- and MDmapA2 lower targeting the *mapA* gene was used (20). For *C. coli*, the primer pair COL3 upper- and MDCOL2 lower targeting the *ceuE* gene was used (20, 23). Cycling conditions for the PCR were 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 90 s at 59 °C and 1 min at 72 °C, with a

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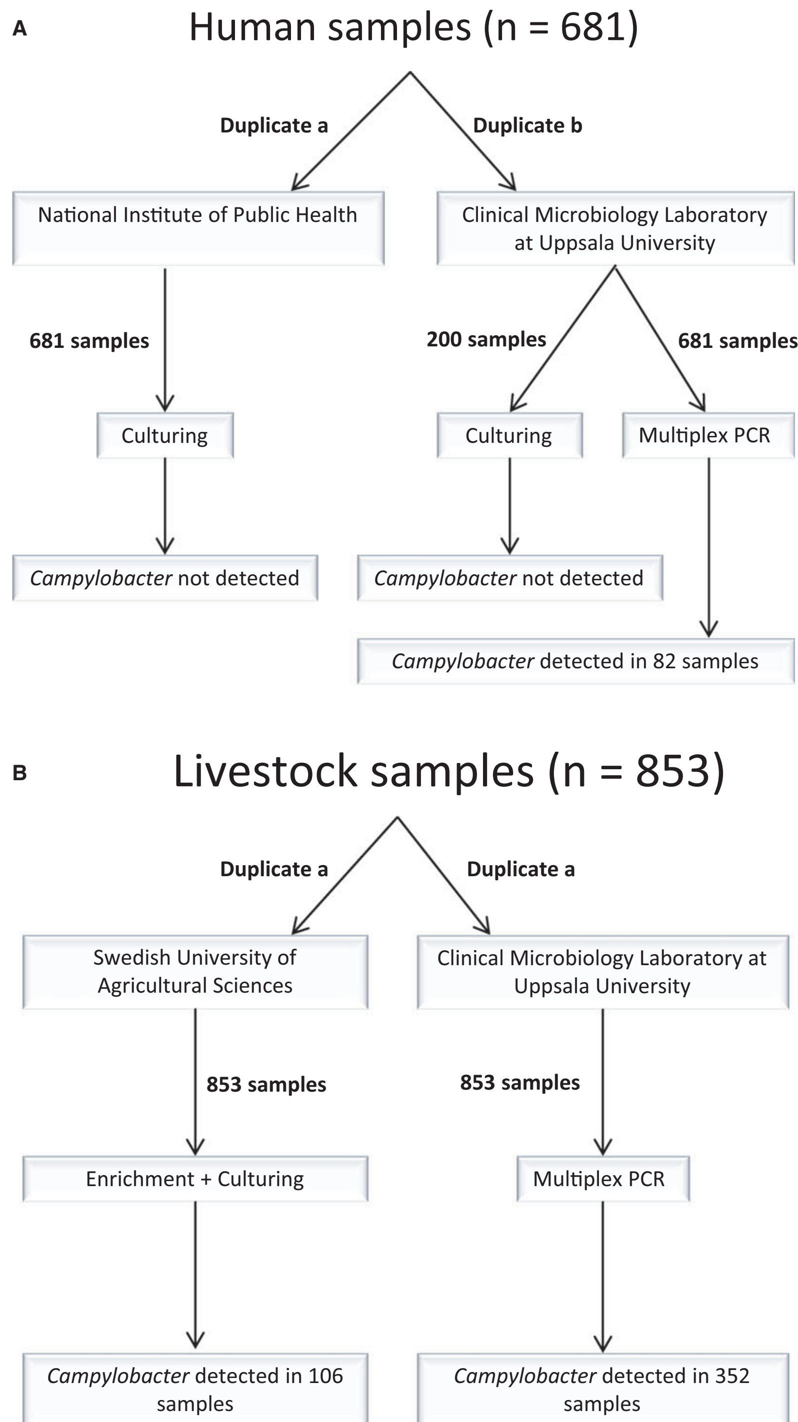

Fig. 2. Diagram of analytical procedure and overall results for detection of *Campylobacter jejuni* and *C. coli* in (A) human faecal samples, and (B) livestock faecal samples, collected in rural Cambodia, 2011–2013.

final extension step of 10 min at 72 °C. The PCR products were separated with gel electrophoresis on 1.5% agarose gel and bands were visualised using GelRed (Biotium, Hayward, CA, USA). The reference strains *C. jejuni* (81-176) and *C. coli* (LMG6440) were used as positive controls and omission of template was used as a negative control.

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Statistical analysis and data management

Statistical analysis of results was performed in SAS for Windows 9.3 (SAS Institute Inc., Cary, NC, USA). Pearson's chi-squared test, or Fisher's exact test when there were less than five observations per group, was used to analyse differences in the proportion of *Campylobacter*-positive samples between children, adult males and adult females; between livestock species and between regions. The statistical significance level was defined as a two-tailed $p \leq 0.05$. Maps were produced in QGIS 2.0.1 (24) with open source base map layers obtained from Open Development Cambodia (25).

RESULTS

Samples were collected and analysed from 681 humans in 269 households and from 853 livestock in 249 households. Both human and livestock samples were obtained from 229 households. None of the human samples tested positive for *Campylobacter* by culture, but by PCR 82 samples (12%) were *Campylobacter* positive; *C. jejuni* was detected in 66 samples (80%) and *C. coli* in 16 samples (20%). Positive samples were detected significantly more often in children below 16 years of age, for whom 19% of samples tested positive for *C. jejuni* or *C. coli*, compared with 8% of samples from adult males ($p = 0.002$) and 7% of samples from adult females ($p < 0.001$) (Table 1 and Fig. 2A). No significant differences in the proportion of *Campylobacter*-positive samples detected by PCR were found between adult males and females or between regions for children and adult females. For adult males, however, the proportion of positive samples was significantly higher in Battambang province (10%) than in Kampong Cham (8%) and Kampot provinces (5%) (Table 2).

Faecal samples from chickens, ducks, pigs, cattle, water buffalo, quail, pigeons and goose were tested by culture and PCR (Table 3 and Fig. 2B). Among the 853 livestock samples, *Campylobacter* were detected by culture in 106 samples (12%); 72 samples (68%) tested positive for *C. jejuni* and 31 samples (29%) for *C. coli*, whereas in three samples (3%) the *Campylobacter* species could not be determined. In the PCR analysis, *Campylobacter* were detected in 352 samples (41%). Among all the positive samples in PCR analyses, *C. jejuni* only was detected in 177 (50%), *C. coli* only in 124 (35%) and both *C. jejuni* and *C. coli* in 51 samples (14%). A comparison of *Campylobacter* species detected in livestock samples by culture and PCR is presented in Table 4. None of the samples collected from buffalo ($n = 25$) and quail ($n = 1$) tested positive for *Campylobacter* by culture or PCR, but one of the pigeon samples ($n = 3$) tested positive for *C. jejuni* by culture and PCR and the only goose sample ($n = 1$) tested positive for *C. jejuni* by PCR, but not by culture. There were significant ($p < 0.001$) differences in the proportion of *Campylobacter*-positive samples in PCR analyses between the four species chickens, ducks, pigs and cattle. However, a regional difference in the proportion of positive samples in PCR analyses was observed only for pigs ($p = 0.02$) among the four main livestock species (Table 2).

Table 1. Detection of *Campylobacter jejuni* and *C. coli* by routine culture and multiplex PCR in faecal samples from children, adult males and adult females in rural Cambodia, 2011–2013

Method	Number (%) of samples positive		
	Children 0–15 years ($n = 272$)	Males > 15 years ($n = 167$)	Females > 15 years ($n = 242$)
Culture: positive for *Campylobacter*	0	0	0
PCR: positive for *C. jejuni or C. coli*	51 (19)	13 (8)	18 (7)
C. jejuni	42 (15)	9 (5)	15 (6)
C. coli	9 (3)	4 (2)	3 (1)

DISCUSSION

To the best of our knowledge, this is the first study to investigate the prevalence of *Campylobacter* concurrently in human and livestock samples originating from the same rural households in Cambodia. Knowing that *Campylobacter* are zoonotic pathogens and that the bacteria can often be recovered from asymptomatic individuals in low-income countries (5), we screened for *Campylobacter* in humans regardless of gastrointestinal symptoms to get an overall prevalence in the rural communities. The observed prevalence of *C. jejuni*/*C. coli* in adults (8% by PCR) was similar to the 10% prevalence described for Tanzanian adults in urban areas screened by culture (26). As expected, children below 16 years were in our study more frequently *Campylobacter*-positive than adults, which might be due to age-related acquisition of protective immunity in individuals living in communities under frequent exposure to *Campylobacter* (2). The observed prevalence of 19% by PCR among children in the present study was similar to that detected by culture in children in poor regions of India, Egypt and Thailand, which ranged from 5.9% to 22% (12, 14, 27). In those studies, *C. jejuni* was also the predominant *Campylobacter* species observed.

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Table 2. Regional comparison of faecal samples testing positive for *Campylobacter jejuni* and *C. coli* by routine culture and by multiplex PCR

Sample type	PCR: positive samples for C. jejuni, C. coli or both				Culture: positive samples for *Campylobacter*			
	KPC, *N* (%)	BTB, *N* (%)	KPT, *N* (%)	p-value[1]	KPC, *N* (%)	BTB, *N* (%)	KPT, *N* (%)	p-value[1]
Children 0–15 years (*n* = 272)	13 (17)	16 (15)	22 (25)	0.18	0	0	0	–
Males >15 years (*n* = 167)	2 (8)	8 (10)	3 (5)	0.05	0	0	0	–
Females >15 years (*n* = 242)	5 (6)	8 (10)	5 (7)	0.58	0	0	0	–
Chickens (*n* = 353)	57 (52)	86 (60)	55 (56)	0.45	31 (28)	27 (19)	29 (29)	0.1
Ducks (*n* = 101)	3 (11)	16 (30)	5 (24)	0.17	2 (7)	2 (4)	1 (5)	0.13
Pigs (*n* = 162)	23 (58)	43 (72)	51 (82)	0.02	2 (5)	5 (8)	4 (6)	0.07
Cattle (*n* = 207)	5 (6)	4 (6)	2 (35)	0.74	1 (1)	1 (1)	0	–

Samples from Kampong Cham province (KPC), Battambang province (BTB) and Kampot province (KPT), Cambodia, 2011–2013.
[1]Chi-squared test.

Table 3. Detection of *Campylobacter jejuni* and *C. coli* by routine culture and multiplex PCR in faecal samples from chickens, ducks, pigs and cattle in rural Cambodia, 2011–2013

Method	Number (%) of samples positive			
	Chickens (*n* = 353)	Ducks (*n* = 101)	Pigs (*n* = 162)	Cattle (*n* = 207)
Culture: positive for *Campylobacter*	87 (25)	5 (5)	11 (7)	2 (1)
C. jejuni	66 (19)	4 (4)	0	1 (0.5)
C. coli	19 (5)	1 (1)	10 (6)	1 (0.5)
C. spp.[1]	2 (1)	0	1 (1)	0
PCR: positive for C. jejuni, C. coli or both[2]	198 (56)	24 (24)	117 (72)	11 (5)
C. jejuni	185 (52)	18 (19)	19 (12)	4 (2)
C. coli	50 (14)	8 (8)	110 (68)	7 (35)

[1]Confirmed *Campylobacter* spp., no species could be determined.
[2]51 samples tested positive for both *C. jejuni* and *C. coli*.

Table 4. Summary of *Campylobacter* species detected by routine culture and multiplex PCR in faecal livestock samples (*n* = 853) in rural Cambodia, 2011–2013

Molecular detection by PCR	Detection by routine culture		
	C. jejuni	*C. coli*	Unspecified[1]
C. jejuni	50	5	0
C. coli	3	14	2
Mixed infection (*C. jejuni* and *C. coli*)	8	9	0
No detection of *C. coli* or *C. jeuni*	11	3	1

[1]Confirmed *Campylobacter* spp., no species could be determined.

The prevalence of *C. jejuni* and *C. coli* in PCR analysis in our study in chickens (56%), ducks (24%), pigs (72%) and cattle (5%) was similar to that found by culture for faecal livestock samples in studies from Vietnam, India and Ethiopia (15, 27, 28). Interestingly, in the present study the prevalence of *C. jejuni* and *C. coli* in chickens was considerably lower than that reported previously for poultry meat sold in wet markets in Cambodia (81%, by culture) (16) or in rural Thai markets (80%, by culture) (29). In our study, *C. jejuni* was the most prevalent *Campylobacter* species in chickens and ducks and *C. coli* was the most prevalent in pigs, which is in line with results from studies performed elsewhere (28, 30, 31). The regional difference in the number of *Campylobacter*-positive samples, identified only for pigs among the four main livestock species screened, suggests a negligible geographical difference in *Campylobacter* prevalence in livestock.

In contrast to the livestock samples, none of the human samples cultured in this study tested positive for *Campylobacter*, despite duplicate analysis of 200 samples in two unalike laboratories applying the

protocols routinely used by the laboratories in *Campylobacter* diagnosis. The difference in the culture results between livestock and human samples is likely due to differences in the handling and culturing procedures. The livestock faecal samples were immediately placed in vials with transport medium, whereas the self-collected human samples were stored for up to 6 h before faecal material was transferred into the vials. With regard to culturing, we decided to use the standard protocols for culturing in the respective veterinary and medical laboratories involved in the study. That is, enrichment was used for livestock but not for human samples which could also explain the different culture results.

Different challenges appear in microbiological diagnosis in high- and low-income countries. The substantially lower detection of *Campylobacter* in human and livestock samples by routine culture compared with PCR observed in this study was most likely due to multiple factors, such as non-optimal conditions for bacterial survival and lower sensitivity of culture in general. It is also important to note that PCR detects bacterial DNA and not only live bacteria, and hence a proportion of the non-culturable samples may contain dead bacteria or levels of viable *Campylobacter* below the detection limit. In settings where people are frequently Campylobacter infected, convalescent-phase samples may also remain positive by PCR while culture is negative. In the rural field conditions described here timely culture was not feasible. Bullman et al. (32) and Platts-Mills et al. (33) have reported on failed growth of *Campylobacter* in samples cultured with a 24-h delay. The 1-day time span from sampling to freezing in this study, most likely reduced the number of viable and cultivable bacteria. Additionally, despite employing a medium successfully used by our research group for long term preservation of *Campylobacter*, storage of samples at −70 °C may have caused further damage as *Campylobacter* cells exposed to freezing have previously been shown less able to recover (34).

The PCR assay used here targeted *C. coli* and *C. jejuni*, the two most frequently isolated *Campylobacter* species in human patients (5). *Campylobacter jejuni* and *C. coli* were also the only *Campylobacter* spp. detected by culture. The culture medium and high incubation temperature applied in this study are known to favour the detection of *C. coli* and *C. jejuni* and fail to support the growth of less common *Campylobacter* spp., including *C. lari*, *C. upsaliensis*, *C. hyointestinalis* and *C. fetus* (32).

The choice of analytical method for *Campylobacter* detection depends on whether the purpose is surveillance, or diagnosis and treatment. PCR has well-known advantages over culture, including considerably faster and more sensitive performance. However, in contrast to some other studies (19, 20, 35), the PCR assay applied here was not 100% sensitive compared with culture in detecting *C. coli*/*C. jejuni*. We suggest that the failure of PCR to detect *C. jejuni* and/or *C. coli* in 15 samples that tested positive by culture was due to insufficient faecal material remaining in the vial once the swab had been removed for culturing purposes.

In this study, we found *Campylobacter*, as detected by PCR, to be highly prevalent in children, ducks, poultry and pigs in rural Cambodian households. Routine culturing was insufficiently sensitive in detecting *Campylobacter* in field samples that had been frozen before analysis. These findings suggest that PCR should be the preferred diagnostic method for detection of *Campylobacter* in humans and livestock where samples need to be frozen and timely culture is not feasible.

We thank the Cambodian families who participated in the study. We also extend our thanks to the commune, district and provincial veterinarians in participating regions for assistance during the field work and to field teams, including staff from NaVRI and students from Preak Leap National School of Agriculture and from the Royal University of Agriculture, both situated in Phnom Penh, Cambodia. Finally, we would like to thank Jamila Mohammad at UU and Mona Fredriksson and Annlouise Jansson at SLU for assistance with sample analyses. The work was financially supported by The Swedish Civil Contingencies Agency (MSB), the Swedish International Development Cooperation Agency (Sida), Sweden, and the Swedish Research Council FORMAS, Sweden. We dedicate this study to our late co-author Prof. Choup Sokheng.

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