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Prevalence and molecular identification of *Campylobacter* species isolates from poultry and humans in Sokoto, Nigeria

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Abstract

Prevalence and molecular identification of *Campylobacter* species isolates from poultry and humans were conducted using culture, biochemical reaction and Polymerase Chain Reaction (PCR) techniques. A total of 798 (506 poultry and 292 human) samples were identified biochemically, out of which 312(39.1%) were positive for *Campylobacter* species. *Campylobacter jejuni*, *C. coli* and *C. lari* had 38 (23.8%) out of 160, 63(39.4%) out of 160, 59(36.9%) out of 160 prevalence rates, respectively in humans while 29(19.1%) out of 152, 79(52.0%) out of 152 and 44(28.9%) out of 152 were the rates for the species in the same order in poultry. *Campylobacter* isolates were kept at -20^{o}C in 15% glycerol and 85% tryptone broth until used while some were identified 24hrs post isolation. Single and multiplex PCR were used to confirm the genus *Campylobacter* and three *Campylobacter* species, respectively. All the 130(100 stored and 30 fresh) isolates were members of the genus *Campylobacter.* The single PCR band view of stored isolates also revealed other bands in addition to 439 bp which is specific for the genus *Campylobacter,* while the fresh isolates had distinct bands at 439bp only. Multiplex PCR revealed 2(6.7%) out of 30 were positive for stored isolates out of 30, 1(50%) each for *C. jejuni* and *C. Coli*. However, 1 of the stored isolate was positive for both spp. On the other hand, 6(20.0%) of out 60 fresh isolates were positive, with 5(83.3%) and 1(16.7%) for *C. jejuni* and *C. coli,* respectively. The possibilities of improper identification using conventional method have been revealed in the study. PCR can identify *Campylobacter* species more accurately than biochemical method, though storage of isolates, integrity of extracted DNA and PCR conditions can affect result. However, the use of both methods should be encouraged in regular and effective surveillance of *Campylobacter* species in poultry and humans.

Keywords: *Campylobacter* species, Humans, Molecular identification , Nigeria, Prevalence, Poultry, Sokoto
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Introduction

Poultry is the most widely domesticated livestock species used for the supply of animal protein to humans. They are kept in confinement or allowed to freely range the farmstead or villages in search of food and at same time contaminate the environment with their faeces (Poultry, 2003). *Campylobacter* species usually colonize the gastrointestinal tract of wide varieties of animals including poultry and establish carrier states for human infection (Butzler, 2004). Contact and handling of poultry in addition to consumption of food and water contaminated with untreated poultry or human waste is believed to have contributed up to 70% of *Campylobacter* related illness among farmers, abattoir workers and children (Deming *et al*., 1987; Coker & Adefeso,

1994; Shane 2000; Coker *et al*., 2002). It is assumed that *Campylobacter* contaminates poultry meat during processing; surviving throughout the food chain supply, constitutes a risk to human health and its elimination in the food chain particularly from chicken product is a challenge (Newell & Fearnley, 2003; Workman *et al*., 2005). Species like *C. jejuni, C. coli* and *C. lari* have been isolated from humans with mild to severe diarrhea and in many food types of animal origin (Jones *et al*., 1931; Altekruse *et al*., 1999; Saenz *et al*., 2000). The absence of food hygiene and sanitation in the abattoirs whereby poultry processors eat uncooked meat during poultry processing, keeping of processed bird on feather wastes and inadequate use of clean water for washing of processed birds may have encouraged the transmission of *Campylobacter* infection to humans (Lawal *et al*., 2013). Other activities like the processing of barbecues and *suya* which are generally accepted delicacy in the study area appear to be a hazardous practice for the infection as they allow easy transfer of bacteria from raw meat to hands and other foods and from these to the mouth.

The public health burdens of *Campylobacter* species still remain unmeasured and information about human infection in the study area is scanty. Few available literature in Nigeria have relied on the use of conventional diagnostic techniques of microscopy, culture and biochemical methods which detect only viable bacteria, and yield isolates that can further be characterized and studied (Engberg *et al*., 2000; Salihu, 2009; Adzitey & Nurul 2011). However, there is need for improvement in the use of molecular techniques which are more sensitive and specific than the conventional method for further identification and studies (Magistrado *et al.,* 2001; Keramas *et al.,* 2004; Nwankwo, 2015).

The purpose of this work was to determine the prevalence of *Campylobacter* species and to identify some of the *Campylobacter* species isolates from poultry and humans using Polymerase Chain Reaction (PCR) technique.

Materials and Methods

Sample

A total of 798 (506 poultry and 292 human) samples were identified biochemically while 130 (100 stored and 30 fresh) isolates were identified with PCR.

Ethical approval

Ethical clearance was obtained from the Ministry of Health, Usman Farouk Secretariat Sokoto, Sokoto state, prior to the study.

The Study area

The study was carried out in Sokoto state, which is geographically located in the extreme northwest of Nigeria. It lies between the latitudes 12^{o}N to 58^{o}N and longitudes 4.8^{o}E to 6.54^{o}E with annual average temperature of 28.3^{o}C. It has 4 agricultural zones and 23 Local Government Areas. It shares boundaries with Zamfara state to the East, Republic of Niger to the North and Kebbi state to the West. The state had a human population of 3.7million people with a population density of 97.7 persons per square kilometer (NPC, 2006) and ranked second to Borno state in livestock production with estimated population of indigenous chicken at 3.4 million (RIM, 1992).

The Study design

The study was carried out with faecal swabs collected from poultry and humans from live bird markets and some hospitals respectively within the selected LGAs of the state. Random and convenience sampling techniques were used in sample collection from poultry and humans respectively. Samples were analyzed biochemically and isolates were kept at -20^{o}C in 15% glycerol and 85% tryptone broth until used. Single and Multiplex PCR were used to confirm the genus *Campylobacter* and three *Campylobacter* species respectively from fresh isolates and those that were stored and recovered after some months in storage.

Sample size determination

The minimum sample size for this study was determined by the formula $N=Z^2p(1-p)/d^2$ (Thrusfield, 1999), where N=sample size or number of individual required; Z is the score for a given interval which is 1.96 (S.E) at 95% confidence interval; p is a known or estimated prevalence; and d is (5%) level of precision. Previous work in the study area obtained *Campylobacter* prevalence rate of 38.8% in birds (Salihu *et al.,* 2009) and 20% as estimated in humans in the study area were used for poultry and humans respectively. With the known prevalence, the minimum calculated sample size (n) required for the study in poultry was 1.96^2 x 0.39x $0.61/0.05^2$ =365, while the minimum sample size required for humans was 1.96^2 x 0.20x0.80/0.05^2=245.

Transportation and processing of samples

The samples were placed in Amies transport media (CMO425, Oxoid) and kept cold in a container with the use of ice block (Butzler, 2004). They were transported to the Veterinary Public Health Laboratory, Faculty of Veterinary Medicine, Usmanu Danfodiyo University, Sokoto for isolation and biochemical identification. They were plated

directly onto selective media; Modified Charcoal Cefaperazone Deoxycholate Agar (mCCDA) and incubated at 42°C for 48hrs under microaerophilic condition generated by Campygen® (Oxoid, BR0056) in the anaerobic jar (Atabay & Corry, 1998; Butzler, 2004). Presumptive *Campylobacter* species were confirmed using standard biochemical procedures which included; Oxidase test (Oxiod Cambridge, United kingdom), Catalase test (3% H_2O_2Sigma Aldrich, St.Louis, Mo,USA), Hydrogen Sulphide production (H_2S) in triple sugar iron (TSI) agar test, Hippurate Hydrolysis test, in addition to sensitivity to Cephalothin, Nalidixic acid and Metronidazole using agar disc diffusion method on mCCDA (Barret *et al*., 1988; On & Holmes, 1992; Quinn *et al*., 1994). Identified isolates were stored at -20°C in a storage media (85% Tryptone broth and 15% Glycerol) at the Applied Biotechnology Division, National Veterinary Research Institute, (NVRI) Vom, Jos Nigeria until used.

DNA extraction

DNA extraction was done using the Qiagen® protocol described in DNeasy Blood and Tissue Handbook (2006). However, the suspension was centrifuged at 20,000 x g for 2 min instead of 300 x g for 5 min as recommended. A total of 130 (100 stored and 30 fresh) isolates were processed.

DNA Amplification

PCR primers (Ingaba Biotec South Africa) which amplify a 439-bp 16S rRNA fragment from the genus *Campylobacter* were used (Moreno *et al*., 2001; Moreno *et al*., 2003) (Table 1).

For *Campylobacter* mono PCR assay, a final reaction volume of 12.5ul was made by the addition of 1.0ul of each sample, 200ng (0.5ul) of each primer, 10mM (0.25ul) of deoxynucleoside triphosphates (dNTPs), 25 mM (0.75 µl) of $MgCl_2$, (2.5 µl) of 10x Greenbuffer, 6.75 µl of H_20 and 5U/ul (0.25 µl) of *Taq* polymerase (NewEngland Biolabs, Inc., Beverly, Mass). The amplification consisted of an initial DNA denaturing step at 95°C for 5 min, followed by 33-cycle reaction (94°C for1 min, 58°C for 1 min, 72°C for 2 min). The cycling included a final extension step at 72°C for 2 min to ensure full extension of the products (Moreno *et al*., 2001).

The DNA amplification was also done for *Campylobacter* species using multiplex PCR. Master mix which contain 200ng (0.25 µl) of primer Forward and 200ng (0.25 µl) primer Reverse for *C. lari*, 200ng (0.5 µl) primer Forward and 200ng (0.5 µl) Primer Reverse for *C. coli*, 200ng (0.25 µl) primer Forward and 200ng (0.25 µl) primer Reverse for *C. jejuni*, in addition to 4.25 µl H_20, 2.5 µl 10x PCR Buffer, 10mM (0.25 µl) dNTPs, 25mM (0.75 µl) $Mgcl_2$, 5U/ul (0.25 µl) Tag polymerase and 2.5 µl DNA (Moreno *et al*., 2001). The above mixture was placed in thermocycler set at initial denaturation temperature of 95°C for 6min, followed by 30 cycles of annealing temp of 95°C for 30 sec, 59°C for 30sec, and 72°C for 30 sec, extension at 72°C for 7 min and finally stops at 4°C (Moreno *et al*., 2001).

Table 1: Genus *Campylobacter* primer and three *Campylobacter* species primers used in PCR analysis

S/NO	*Campylobacter* genus/species	Primers names	Primer sequence 5´to 3´	References
1	Genus *Campylobacter*	Forward primer CAM 220	GGTGTAGGATGAGACTATATA	Moreno *et al*., 2003
		Reverse primer CAM 659	TTCCATCTGCCTCTCCCY	
2	*C. jejuni*	Forward primer	ACTTCTTTATTGCTTGCTGC	Moreno *et al*., 2001
		Reverse primer	GCCACAACAAGTAAAGAAGC	
3	*C. coli*	Forward primer	GTAAAACCAAAGCTTATCGTG	Moreno *et al*., 2001
		Reverse primer	TCCAGCAATAGTGTGCAATG	
4	*C. lari*	Forward primer	TAGAGAGATAGCAAAAGAGA	Moreno *et al*., 2001
		Reverse primer	TACACATAATAATCCCACCC	

Source: Ingaba Biotec, South Africa

Table 2: Prevalence of *Campylobacter* species from humans and poultry

Sample source	Total sampled	Total + (%)	*C. jejuni* (%)	*C. coli* (%)	*C. lari* (%)
Humans	292	160(54.8)	38(23.8)	63(39.1)	59(36.9)
Poultry	506	152(30.0)	29(19.1)	79(52.0)	44(28.9)
Total	798	312(39.1)	67(21.5)	142(45.5)	103(33.0)

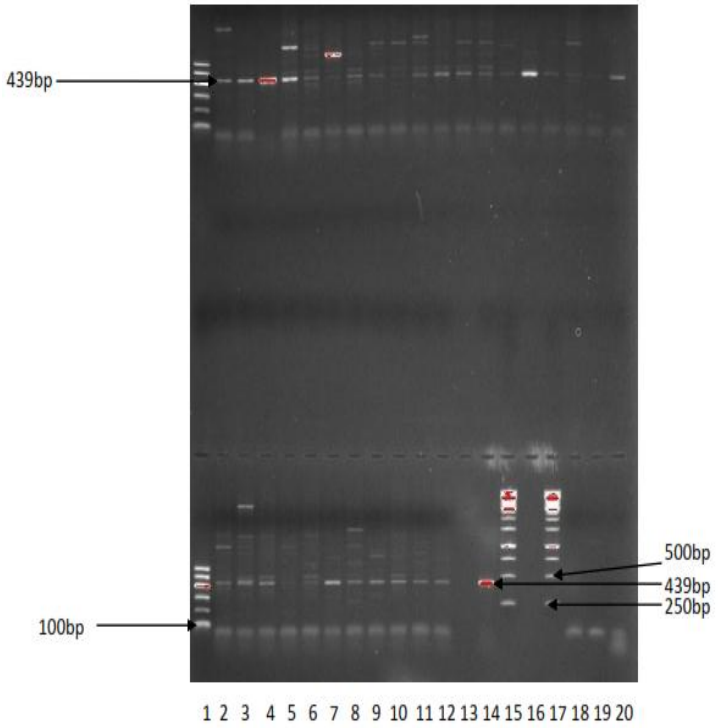

Figure 1: A gel picture of Single PCR detection of the genus *Campylobacter* (Stored samples 1-30) Lane 1, 100bp DNA ladder marker;Lane 15 &17, 250bp DNA ladder marker;Lane 14, *Campylobacter* genus positive control@ 439bp;Lane 13, negative control (Lower view)

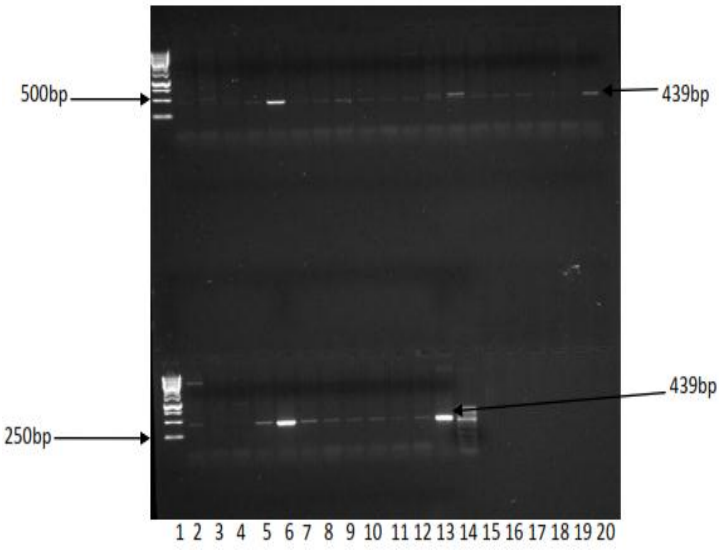

Figure 2: A gel picture of Single PCR detection of the *genus Campylobacter* (Fresh samples 1-30). Lane 1, 250bp DNA ladder maker; Lane 13, *Campylobacter* positive control (Lower view); Lane 2-17 and 20, (samples1-16 and 19) had distinct bands at 439bp and were positive for *Campylobacter* (Upper view); Lane 2, 5-12, (samples 20, 23-30) had distinct bands at 439bp and were positive for *Campylobacter* (Lower view); Lane 6, 9, 14 and 20 (Upper view) and 5, 6, 7, 8, 10 12 (Lower view) were clearer

Gel Electrophoresis

Gel electrophoresis was carried out in 1.5g of agarose powder in trisboric Acid/EDTA (TAE) buffer which contained 5 µl of ethidium bromide (5mg/ml) at 120 volts for 40mins. The gel pictures were viewed and documented. Band views of 439, 323, 126 and 251 base pairs (bps) for genus *Campylobacter*, C. *jejuni*, C. *coli* and C. *lari* respectively were determined. A DNA ladder and positive control were included during electrophoresis.

Results

Campylobacter jejuni strains produced grey, moist flat spreading colonies. *Campylobacter coli* strains tends to be creamy-grey in colour, moist, slightly raised (Oxoid manual). A total of 312 out of 798 (506 poultry and 292 human) (39%) were positive for *Campylobacter* species after biochemical analysis. *Campylobacter jejuni*, *C. coli* and *C. lari* had 38 out of 160 (24%), 63 out of 160 (39%), 59 out of 160 (37%) prevalence rates respectively in humans while 29 out of 152 (18%), 79 out of 152 (53%) and 44 out of 152 (28%) were for the species in the same in poultry (Table 2). A total of 130 (100 stored and 30 fresh) isolates were used in the single PCR while 30 out of the 100 (30%) stored and 30 fresh isolates were used in the multiple PCR. Single PCR identification for both stored and fresh isolates for the genus; *Campylobacter* was 100% positive (Table 3). However, by single PCR, the stored isolates revealed bands at 439 bp which is specific for the genus *Campylobacter* in addition to other unknown multiple bands (Figure 1). On the other hand, the fresh isolates revealed distinct band with sizes of 439 bp with non-specific amplicons (Figure 2). Multiplex PCR analysis for three *Campylobacter* species revealed 2 (7%) positive for *Campylobacter* species in the stored isolates (Table 4). One of the two positive isolates had two bands at 323 and 126 base pairs (bp) which are specific for *C. jejuni* and *C. coli* respectively while the other had band at 126 bp (Figure 3). However, the two isolates were identified biochemically as *C. jejuni* and *C. lari* respectively. Furthermore, multiplex PCR of the fresh isolates with clear bands revealed 5 (17%) and 1 (3%) positive for *C. jejuni* and *C. coli* respectively (Figure 4) while no species was identified in isolates with faint bands.

Table 3: Single PCR of Campylobacter genus in stored and fresh isolates from poultry and humans

Isolates	Total analyzed	Total + (%)	Total – (%)
Stored	100	100 (100)	0
Fresh	30	30 (100)	0
Total	130	130(100)	0

Table 4: Multiplex PCR for detection of three *Campylobacter* species in stored and fresh isolates from poultry and humans

Isolates	Total analyzed	Total + (%)	*C.jejuni* (%)	*C.coli* (%)	*C.lari* (%)
Stored	30	2 (6.7)	1 (50.0)	2 (100.0)	0
Fresh	30	6 (20.0)	5 (83.3)	1(16.7)	0
Total	60	8 (13.3)	6 (75.0')	3(37.5)	0

Note: One stored isolate was positive for both *C. jejuni* and *C .coli*

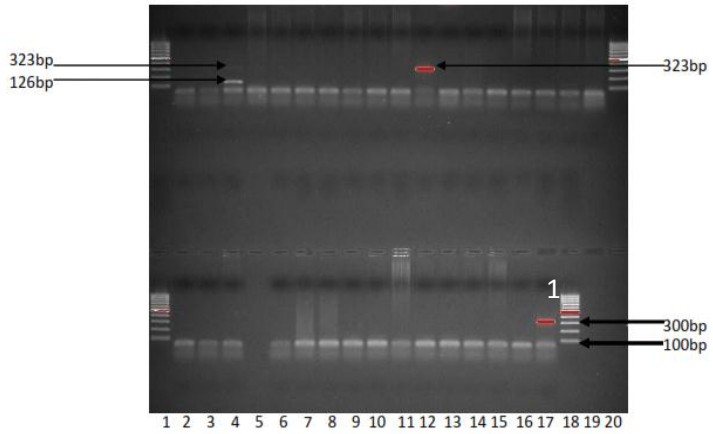

Figure 3: A gel picture of Multiplex PCR detection of *Campylobacter* species (Stored samples 1-30). Lane 1 and 20. 100bp DNA ladder marker; Lane 12, *C. jejuni* positive control. 323 bp (upper view); Lane 5, negative control (lower view);Lane 4, (sample 17) had two bands at 126bp and 323bp specific for *C. coli* and *C. jejuni* respectively (Upper view) Lane 17, (sample 22) had one band at 323bp specific for *C. jejuni* (Lower view)

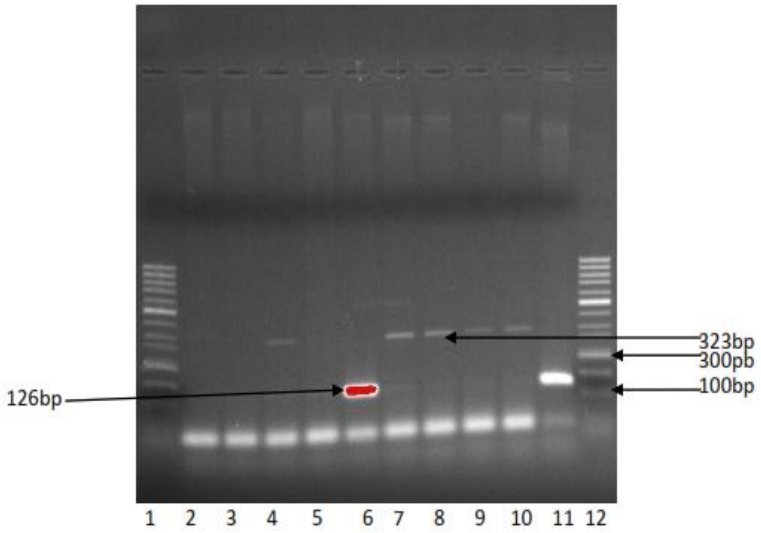

Figure 4: A gel picture of Multiplex PCR detection of *Campylobacter* species fresh samples (Genus; *Campylobacter* with clear bands) Lane 1 and 12, 100bp DNA ladder marker; Lane Lane 2 and 3, (samples 5 and 8) were negative for *Campylobacter species*. Lane 4 (sample 19) was positive for *C. jejuni* Lane 5, (sample 23) was negative for *Campylobacter* species Lane 6, (sample 24) was positive for *C. coli*; Lane 7-10, (samples 25-28) were positive for *C. jejuni*; Lane 11, *C. coli*

Discussion

Campylobacter species have been detected with different prevalence rates in poultry and humans in Sokoto state, Nigeria. Poultry may have contributed to the high prevalence in humans as birds are usually raised at backyard of different households and presented for sale and slaughter at live bird markets where they share same environment with bird sellers. Humans in the study area are at risk of gastroenteritis since the most common bacterial causes of infectious intestinal disease, *C. coli* and *C. jejuni* have been identified (Altekruse *et al*., 1999; Coker *et al*., 2002; Uaboi-Egbenni *et al*., 2008). The *Campylobacter* species prevalence rates in humans were in agreement with the rates recorded in the work of Gwimi *et al.* (2015) in a neighboring state of Kebbi, Nigeria where *C. coli* was higher in occurrence than other species. However, the findings disagreed with studies that revealed *C. jejuni* as the most common species of *Campylobacter* in humans in Spain and Nigeria (Saenz *et al*., 2000; Ohanu & Offune 2009). On the other hand, the higher prevalence rate of *C. coli* than *C. jejuni* agreed with the rate in the work of Wieczoreck *et al*. (2012) in Poland but is not in concordance with studies where *C. jejuni* was more than other species in poultry in Nigeria and Washington, D.C (Cuiwei *et al*., 2001; Salihu *et al*., 2009). Differences and similarities in

positive control

prevalence rates have been associated with the sample size, geographical location and season of isolation (Mary *et al*., 2004; Nwankwo *et al*., 2016). The use of Polymerase Chain Reaction (PCR) as a very rapid and highly sensitive molecular diagnostic technique than the conventional method in the detection of *Campylobacter* infections in poultry and humans has not been proven satisfactorily in the study (Magistrado *et al.,* 2001; Keramas *et al*., 2004). However, the result of the stored isolates which did not give good picture of DNA band compared to that of fresh isolates suggested the possibilities of contamination during storage. Since isolates are often stored for use in PCR when the necessary reagents are not available, an optimal temperature of -80^{o}C has been suggested for their survival and conservation. Studies have showed that PCR can be used to directly detect *Campylobacter* spp., in the presence of other contaminating bacteria (Magistrado *et al*., 2001; Abulreesh *et al.,* 2010). The identification of *C. jejuni* and *C. coli* in one isolate and *C. coli* in another isolate which were biochemically identified as *C. jejuni* and *C.lari* respectively has further revealed the possibilities of contamination and improper identification or wrong interpretation of conventional phenotypic result (Adzitey & Corry, 2011). This observation was in agreement with the work of Siemer *et al. (*2005) where 12% isolates originally identified as *C. coli* by phenotypic method were actually *C. jejuni* with the use of molecular method. The bright and faint bands showed in the multiplex PCR of fresh samples might be a reflection of the DNA content and the result of samples with faint bands is an indication that viable *Campylobacter* species could not sometime be detected by PCR due to the decrease of DNA content. However, the integrity of recovered DNA can be validated on a 2% gel before use in PCR. Furthermore, the sensitivity and performance of PCR analysis can also be inhibited by components of DNA extraction solution, concentration of the PCR mixtures (primers, DNA templates, dNTP's an Mg^{2+}), temperature and cycling conditions (Rossen *et al*., 1992; Linton *et al*., 1997; Wilson, 1997; Wassenaar & Newell 2000).

The higher isolation rate of *C. jejuni* than *C. coli* and no *C. lari* in the Multiplex PCR of fresh samples was in agreement with similar result obtained with the use of the same diagnostic technique for *Campylobacter* species (Boonmar *et al*., 2007). However, the challenges of contamination can be resolved with a better choice of molecular method like Real- time PCR which also has the benefit of quantitative application (Cobo, 2012). The use of both the conventional and molecular methods are essential in regular and effective surveillance of *Campylobacter* species since isolates that were identified biochemically, can be confirmed and studied further with molecular technique (Adzitey & Corry 2011; Adzitey *et al*., 2013).

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