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# Molecular Identification of *Campylobacter jejuni* and *Campylobacter coli* Isolated from Small-Scale Poultry Slaughterhouse in Lima, Peru

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**Abstract:** A total of 96 samples (3 scalding water, 3 final washing water, 30 non-eviscerated, 30 eviscerated and 30 cecal content) from three small-scale broiler slaughterhouses were evaluated. Bacteriological test was performed with mCCDA medium and positive samples were confirmed by PCR assays using 16S rDNA, *hip*O and *asp* primers to *Campylobacter* spp., *Campylobacter jejuni* and *Campylobacter coli* respectively. Bacteriological test showed the presence of *Campylobacter* spp. in 60 samples. However, according to the PCR assays, only 19 samples were confirmed as positive. Non-eviscerated and eviscerated carcasses had fewer numbers of samples positive for *Campylobacter* spp. (63 and 50%, respectively) than the cecal content samples (77%). No positive were found in the scalding and the final washing water samples. No differences (P>0.05) were observed between PCR and biochemical tests for *Campylobacter jejuni* and *Campylobacter coli* identification. Chicken meats from small-scale slaughterhouses in Lima, Peru are potential reservoirs of *Campylobacter jejuni* and *Campylobacter coli* and this contamination was associated to some deficiencies in slaughter process, principally during the evisceration process. The method presented in this paper has shown to be suitable determination of *Campylobacter* species in faecal, meat and water samples.

**Key words:** Bacteriological, chicken, evisceration, molecular, slaughterhouse

## INTRODUCTION

*Campylobacter* spp. and in particular the thermotolerant species, *Campylobacter* jejuni and *Campylobacter coli*, are the most common pathogens in diagnosed human *Campylobacter* infections (Tam *et al.*, 2003). There is strong evidence suggesting that poultry and poultry products are main sources of thermophilic *Campylobacter* spp. infection in humans (Silva *et al.*, 2011). Although all commercial poultry species can become carriers of *Campylobacter*, the greatest current risk to human health is posed by contaminated chicken (ACMSF, 2005; Humphrey *et al.*, 2007).

*Campylobacter* may be transferred to humans indirectly through the ingestion of contaminated water or food and to a minor extent by direct contact with contaminated animals or animal carcasses (Figueroa *et al.*, 2009). In developed countries, risk factors associated with food include occupational exposure to farm animals, consumption of poultry meat and unhygienic food preparation practices as important potential sources of infection in humans (Corry and Atabay, 2001).

Many reports around the world have demonstrated the relationship between strains isolated from human infections and chickens, mainly in developing countries. During the last decade of the 20th century several studies have been conducted demonstrating the presence of *Campylobacter* species in various stages of poultry production, *Campylobacter* spp. has been isolated in 77% of retail poultry meat sold in Bangkok (Rasrinaul *et al.*, 1988). In Mexico City, a survey of ready-to-eat roasted chickens showed that such product was contaminated with *Campylobacter* spp. (Quinones-Ramirez *et al.*, 2000) In Santiago de Chile, poultry slaughterhouses had the greatest risk of contamination during evisceration, such risk decreasing after chilling process (Figueroa *et al.*, 2009).

In Peru, Tresierra *et al.* (1995) found that chicken is the largest reservoir (54%) of *Campylobacter* species, *C. jejuni* being the most frequent (23,6%) in Iquitos city. In another study Perales *et al.* (2002) reported that 13.3% of diarrhea in 2 year-old children with episodes of diarrhea were infected with *Campylobacter* spp. in Lima

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city and Oberhelman *et al.* (2006) between free-range chicken and *Campylobacter* infection in humans from a Peruvian periurban shantytown. In spite of importance of *Campylobacter* species, there is no information about the prevalence of this microorganism in Peruvian poultry slaughterhouses.

In the last year, the Peruvian citizens increased their consumption of chicken meat as a result of reduced fish supplies and higher prices of other sources of meat (MINAG, 2010). Peruvian legislation defines the processes of poultry meat and poultry meat products for human consumption, specifying proper technical conditions and equipment used for such purpose in order to guarantee health safety and chicken meat quality (MINAG, 2007). However, small-scale poultry slaughterhouse-popularly known as "peladurias" - which process no more than 1000 birds per day, perform all processing steps in manual and rudimentary conditions. Frequently, hygienic conditions are poor and contribute to increase risk of carcasses contamination. For these reasons, the aim of this study was to determinate by molecular techniques the presence of *Campylobacter jejuni* and *Campylobacter coli* in broiler carcass from small-scale slaughterhouses in Lima, Peru.

## MATERIALS AND METHODS

A total of 96 samples were evaluated. Samples were collected during a 5-month period from three small-scale chicken slaughterhouses, registered and authorized by the National Service of Agricultural Sanity (Servicio Nacional de Sanidad Agraria-SENASA) in Lima, Peru. Thirty different chicken carcasses (10 non-eviscerated, 10 eviscerated and 10 cecal contents), one sample of scalding water and one of final washing tank sample were collected from each slaughterhouse. The carcasses were subjected to a Whole Carcass Rinse (WCR) procedure described by Kuana *et al.* (2008) as follows: Carcasses were transferred into clean plastic bags and 150 mL of 1% peptone solution was added. Bagged carcasses were shaken for 1 min and the solution was transferred to sterile flasks. The cecal content was aseptically removed from each chicken by swab and placed into sterile plastic bags. Water samples (1000 mL) from the scalding and the final washing tank were collected in sterile plastic bottles during the slaughter process. All samples were kept at 4±2°C and transported to the laboratory within one hour after sampling.

Microbiologic assays were performed in the Laboratory of Public and Environmental Health, Universidad Nacional Mayor de San Marcos (Lima, Peru). Rinse fluid (0.1 mL) from each carcass was plated onto *Campylobacter* blood-free selective medium (modified CCDA-Preston, mCCDA; CM0739, Oxoid, Hampshire, UK) containing CCDA selective supplement (SR0155, Oxoid) and incubated at 42±1°C for 22±2 h under microaerobic conditions (5% $O_2$, 10% $CO_2$ and 85% $N_2$) obtained by CampyGen Compact (CN0020C, Oxoid) (Isohanni and Lyhs, 2009; Lindmark *et al.*, 2009). The cecal samples were opened and contents were streaked directly on the same medium previously described (Alter *et al.*, 2011).

All suspected *Campylobacter* colonies which were small sized, flat, low convex, mucoid gray, glossy, sticky, swarming with metallic sheen appearances in the medium, Gram-negative staining were sub-cultivated onto blood agar plates containing 5% sheep blood, under microaerobiosis conditions, at 42°C for 72 h. All *Campylobacter* strains were frozen at -80°C in Brain Heart Infusion with 17% (v/v) glycerol, awaiting biochemical tests and PCR assays.

Positive samples from bacteriological isolation were tested for catalase, oxidase, hippurate hydrolysis and for susceptibility to nalidixic acid and cephalothin. Hippurate positive isolates were identified as *C. jejuni* and nalidixic acid susceptible and hippurate negative isolates as *C. coli* (Hariharan *et al.*, 2009).

Suspected colonies from selective media and biochemical tests were confirmed by PCR assays in the Laboratory of Virology, Faculty of Veterinary Medicine, Universidad Nacional Mayor de San Marcos (Lima, Peru). Total DNA was extracted with a standard phenol: Chloroform (v:v) procedure followed by precipitation of DNA by ice-cold ethanol. DNA was resuspended in a 60 µL of nuclease free water and stored at -70°C until further use. PCR assays were performed using the modified protocol of Persson and Olsen (2005). Briefly, a total reaction volume of 25 µL containing 1x of Platinum® PCR Supermix (20 mM Tris/HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTP, 20U Platinum *Taq* Polymerase) (Invitrogen), 0.4 µM *asp* (Linton *et al.*, 1997), 0.2 µM hipO and 0.05 µM 16S rDNA primers (Table 1) were prepared as a master mix while 10% of total reaction volume was used as template for each suspected positive isolation.

PCR amplification was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research, California, USA) using an initial denaturation step of 94°C for 6 min, followed by 35 cycles of denaturation at 94°C for 50 s; annealing at 57°C for 40 s and extension at 72°C for 50 s. After the last cycle, a final extension step of 72°C for 3 min was added (Linton *et al.*, 1997). PCR products were analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide. Band visualization was carried out on a UV transilluminator (Ultra Lum, USA) and photographed for further analysis.

The prevalence of *Campylobacter* species was calculated using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Chi-square ($X^2$) tests were used to determine statistically significant differences between the biochemical test and PCR assay.

Table 1: Primers used in the identification of *campylobacter* spp. *Campylobacter coli* and *campylobacter jejuni* in PCR assays

| Primer | Nucleotide sequence 5'-3' | Origin | Target detected |
|---|---|---|---|
| CC18F | F: GGTATGATTTCTACAAAGCGAG | Linton *et al.* (1997) | *Campylobacter coli* |
| CC519R | R: ATAAAAGACTATCGTCGCGTG | | |
| hipO-F | F: GACTTCGTGCAGATATGGATGCTT | Persson and Olsen (2005) | *Campylobacter jejuni* |
| hipO-R | R: GCTATAACTATCCGAAGAAGCCATCA | | |
| 16S-F | F: GGAGGCAGCAGTAGGGAATA | Persson and Olsen (2005) | *Campylobacter* spp. |
| 16S-R | R: TGACGGGCGGTGAGTACAAG | | |

## RESULTS AND DISCUSSION

Bacteriological test confirmed the presence of *Campylobacter* spp. on chicken carcasses, cecal contents and in final washing water. After the bacteriological examination 60 samples were identified as presumable *Campylobacter* spp. According to growth on mCCDA medium; final washing water and cecal content samples showed the highest percentage (100 and 77%, respectively). Positive samples in the bacteriological examination were evaluated by a PCR assays; the results showed that 19 samples were compatible with *Campylobacter* spp.; in this case cecal content and eviscerated carcasses had the highest percentage. On the other hands, no positive samples were identified in both types of water samples by PCR assay (Table 2).

The high number of positives in the cecal content (23/30), suggests that the origin of chicken contamination occurred at the farm. In Peru, a few studies about the presence of *Campylobacter* species in chicken farms had been reported (Tresierra *et al.*, 1995). However, the presence of high rates of *Campylobacter* spp. in the large intestine is known, Corry and Atabay (2001) reported that $10^5$-$10^9$ CFU $g^{-1}$ of *Campylobacter* spp. are commonly observed in the intestinal content, emphasizing that levels above $10^{12}$ CFU $g^{-1}$ can be found in the cecal contents.

*Campylobacter* spp. was detected in carcasses; which can be attributed to rudimentary processing and poor hygienic conditions in small-scale slaughterhouses, some of these events were: no stunning procedures, slaughter was basically manual, no chilling process, final washing was performed in a small water tank full of tap water at room temperature. Johannessen *et al.* (2007) determinated that the contamination of chicken meat occurs during slaughter and processing, either at slaughter, when carcasses of colonized birds may become contaminated by faecal matter, or while passing down the line due to cross-contamination. As expected, non-eviscerated carcasses had the lower percentage of *Campylobacter* spp. The presence of this microorganism in this samples may be attributed to carcass contamination after scalding when the follicles remain open allowing some microorganisms to be retained until follicles close completely during chilling (Berndtson *et al.*, 1992; Corry and Atabay, 2001). We considered the evaluation of non-eviscerated carcasses because this commercial presentation is the most requested by local markets which sell them separately from viscera and consumers associate it with freshness.

We observed an increase of positive carcasses to *Campylobacter* spp. after the evisceration; this might be attributed to some deficiencies in evisceration procedures. In the Peruvian poultry industry, manual and mechanical methods are employed for the evisceration process; however, in small-scale slaughterhouses manual methods are more frequently used and preferred than mechanical ones because its cost far less relative to acquisition of machines. Figueroa *et al.* (2009) related that in some cases incorrect procedures make possible a viscera rupture, leading inevitably to contamination of equipment, working surfaces, water and meat, increasing the opportunities for *Campylobacter* cross contamination. Pandey and Bawa (2010) determinated manual techniques are preferred and in widespread use because of limitations in using machines. Automatic machines for evisceration are usually species specific; the species involved and uniformity in size are very important for proper operation. In manual methods the entire process is performed on a table and the bird is passed along from one operator to another until it is finished.

No positive samples were observed from scalding water by both assays, this fact can be explained due to the scalding process being performed in small tanks with water temperatures between 60 and 70°C. Based on the work of Osiriphun *et al.* (2012) *C. jejuni* is sensitive to thermal conditions being destroyed at temperatures of 55 and 60°C with immersion times between 2-4 min. Similarly, no positive results were obtained from final water washing by PCR assay. Controversially, two samples were positive to *C. coli* by biochemical tests. These results can be explained because nalidixic acid test is positive to nalidixic acid-sensitive *Campylobacter* species (e.g.: *Campylobacter lari*) being difficult to distinguish from *C. jejuni* and *C. coli* (Duim *et al.*, 2004). Carcasses are commonly washed using chlorinated water to remove contamination such as blood, tissue fragments and faeces as part of regular processing procedures (Keener *et al.*, 2004). Bashor *et al.* (2004) determinated that the washing systems used for the inside and outside surface cleaning of chicken carcasses have limited effectiveness for *Campylobacter* removal.

Table 2: Result of *campylobacter* spp. In bacteriological and PCR assay in samples collected from small-scale slaughterhouses en lima, peru

| Samples | No. of samples* | No. of positive to *Campylobacter* spp. | |
|---|---|---|---|
| | | Bacteriological test | PCR assay |
| Non-eviscerated carcass | 30 | 19 (63%) | 3 (10%) |
| Eviscerated carcass | 30 | 15 (50%) | 7 (23%) |
| Cecal content | 30 | 23 (77%) | 9 (30%) |
| Final washing water | 3 | 3 (100%) | 0 |
| Scalding water | 3 | 0 | 0 |
| Total | 96 | 60 (63%) | 19 (20%) |

*Total samples evaluated for each point

Table 3: Result of *campylobacter jejuni* and *campylobacter coli* by biochemical test and PCR assay obtained from positive bacterial isolations of small slaughterhouses in lima, peru

| Samples | No. of samples* | Biochemical test | | PCR assay | |
|---|---|---|---|---|---|
| | | *C. jejuni* | *C. coli* | *C. jejuni* | *C. coli* |
| Non-eviscerated carcass | 19 | 4 | 3 | 3 | 0 |
| Eviscerated carcass | 15 | 2 | 1 | 2 | 5 |
| Cecal content | 23 | 8 | 3 | 5 | 3 |
| Final washing water | 3 | 0 | 2 | 0 | 0 |
| Scalding water | 0 | 0 | 0 | 0 | 0 |
| Total 60 | 14 | 9 | 10 | 8 | |

*Positive samples to *Campylobacter* spp. by bacteriological tests

Table 4: Comparatione of PCR assay and biochemical test results by chi-square test

| Detection method | No. of samples tested* | *C. jejuni* | | *C. coli* | |
|---|---|---|---|---|---|
| | | + | - | + | - |
| PCR | 60 | $10^{a}$ | $50^{a}$ | $8^{a}$ | $52^{a}$ |
| Biochemistry | 60 | $14^{a}$ | $46^{a}$ | $9^{a}$ | $51^{a}$ |

*Positive samples to *Campylobacter* spp. by bacteriological tests

[a,b]Different letters show significant difference in the same column (P<0.05)

Since *Campylobacter* spp. is a common inhabitant of the intestinal tract of chickens, the major risk of meat contamination occurs while slaughtering and evisceration, the main goal to control bacterial contamination of chicken carcasses during processing is to minimize the spreading of faecal material (FAO, 2003; Nauta *et al*., 2009). It is important to know that the accidental ingestion of one drop of raw chicken juice can easily constitute an infectious dose and the infections can occur during improper handling of raw chicken carcasses, by consuming insufficiently cooked chicken meat and via cross-contamination of other types of food by contact with knives or cutting boards used to prepare raw chicken (Newell and Wagenaar, 2000).

Eviscerated and non-eviscerated carcasses showed contamination with *Campylobacter jejuni* and *Campylobacter coli* species by biochemical tests and PCR assays (Table 3). Both tests found that *Campylobacter jejuni* was most prevalent (10% for PCR and 14% for biochemical), being frequently found in the cecal content samples. *Campylobacter jejuni* is related with human Campylobacteriosis. Mor-Mur and Yuste (2010) reported this species as the implicated in clinical diagnosis of sporadic bacterial human gastroenteritis. On the other hand, Fernández (2011) observed that in South America, *Campylobacter coli* has been isolated most frequently from water, poultry meat and faeces, representing about 25% of human diarrhea cases. Due to high consume of chicken meat in Peru, it is very important that the poultry industry and government authorities have adequate control in the slaughterhouse process. Although Peruvian inspection programs oversee the production and marketing of chicken, some abattoirs, such as small-scale, escape these controls and could be the starting point for several problems related to poor hygiene in the chicken slaughter process. Although the conventional culture methods are hinder because of the fastidious nature of *Campylobacter* species (slow growing features with specific requirements related to incubation atmosphere), these are still preferred by several laboratories and considered a useful screening tool for the identification of this microorganism (On, 1996). In our study, the positive samples from the bacteriological test were evaluated by PCR and biochemical test but no significant difference (Chi-square test, P>0.05) was observed between both tests (Table 4). These results indicate that both techniques can be successfully used to detect Campylobacter spp. Nevertheless Leblanc-Maridor *et al*. (2011) and Singh *et al*. (2011) indicate that a PCR assay

is the best method for identification and differentiation of *Campylobacter* species due to its efficiency, speed and reliability in various substrates and processing a large number of samples at one time. On the other hand, Steinhauserova *et al.* (2001) concluded that there seems to be no single ideal method that could be used in practice for secure identification of all *Campylobacter* spp. and it is necessary to choose combinations of available techniques to compensate for weak points of individual methods.

**Conclusion:** Chicken meats from small-scale slaughterhouses in Lima, Peru, are potential reservoirs of *Campylobacter jejuni and Campylobacter coli*. Meat contamination was associated to some deficiencies in the slaughter process in this kind of location, especially during the evisceration processes. It is necessary to improve such process and implement sanitation to reduce the risk of contamination. Bacteriological and PCR assays can be useful to determinate *Campylobacter* spp. Further studies should be carried out inside the poultry farms and also in medium and large scale poultry slaughterhouses in order to determine the true prevalence and implication of *Campylobacter* species in public health in Lima, Peru.

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