Investigation of emerging Campylobacters in chicken meat products in Italy

A. DE CESARE*, G. MANFREDA, V. BONDIOLI and A. FRANCHINI

Department of Food Science, Alma Mater Studiorum-University of Bologna
Via San Giacomo 9, 40126 Bologna, Italy
*Corresponding author: adecesare@disa.unibo.it

The Campylobacter species isolated from chicken meat most frequently responsible for human campylobacteriosis are \( C. \) jejuni and \( C. \) coli. However, other emerging species, such as \( C. \) upsaliensis, \( C. \) helveticus, \( C. \) hyointestinalis and \( C. \) lari might be transmitted to humans through poultry meat based products. The aims of this study were to investigate the presence these emerging Campylobacters on 222 broiler carcasses and 243 chicken meat products using the Cape Town and the CAMPYCHECK protocols, respectively. The Campylobacter prevalence estimated on broiler carcasses and chicken meat products were 100 and 46.1%, respectively. However, according to the identification results, collected testing as much as 950 colonies isolated from the positive samples, any emerging Campylobacter species seem to contaminate Italian broilers as well as chicken meat products. This study was financed within the EC FP5 CAMPYCHECK project QLK1 CT 2002 02201 (www.campycheck.org).

Keywords: emerging; Campylobacters; chicken meat; Italy

Introduction

Campylobacter species are recognised world-wide as one of the most frequent causes of gastrointestinal disease (Bereswill and Kist, 2003).

When the diagnosis of infection is based exclusively upon isolates obtained on culture media containing blood, detoxifying charcoal and selective antibiotics it is found that approximately 85 to 95, and 5 to 10% of Campylobacter infections are caused by \( C. \) jejuni subsp. \( jejuni \) and \( C. \) coli, respectively. However, it has been suggested that other campylobacteria may be significantly under-diagnosed as causes of gastrointestinal disorders due to inappropriate isolation and identification methods (Engberg et al., 2000).

As an alternative to the selective media it can be used the procedure named “Cape Town Protocol” involving the passage of motile campylobacteria through a membrane filter onto a non-selective growth agar medium, incubated in an \( \text{H}_2 \)-enriched microaerobic atmosphere (Le Roux and Lastovica, 1997). This protocol was the only one available to test emerging Campylobacters before the set up of a new method during the EU founded project named CAMPYCHECK, regarding the improvement of physiological, immunological and molecular tools for the recovery and identification of emerging Campylobacteriaceae in the food and water chain.

The phenotypic methods to speciate Campylobacters are limited by their asaccharolytic and fastidious growth characteristics, which restrict the number of differentiation biochemical testes (On, 1996). Additional difficulties are represented by the lack of standardised methods, subjective interpretation of results and existence of biochemically atypical strains. Consequently, there is considerable interest in molecular approaches for Campylobacter identification. Manfreda et al. (2003) improved a multiplex PCR (mPCR) protocol to identify both \( C. \) jejuni and \( C. \) coli in the same reaction. Linton et al. (1996) set up a mPCR to identify \( C. \) upsaliensis, \( C. \) helveticus, \( C. \) hyointestinalis and \( C. \) lari, amplifying different regions of the 16S ribosomal RNA genes.
The main goals of this study were to apply the Cape Town protocol to isolate Campylobacter on broiler carcasses and the CAMPYCHECK protocol to test poultry meat products. Then to identify a certain number of strains with different morphology by PCR to evaluate the presence of *C. upsaliensis*, *C. helveticus*, *C. hyointestinalis* and *C. lari*.

**Materials and methods**

A total of 222 broiler carcasses were collected in the same slaughterhouse, between October 2003 and September 2004, after the air cooling operation and tested for the presence of Campylobacter spp using the Cape Town protocol. All carcasses were placed into sterile plastic bags and rinsed with 300 or 200 ml of sterile water according to their weight. Then, carcasses were manually shaken for 1.30 min and the rinse suspensions were aseptically transferred to new sterile bags chilled at 4°C to be transferred to the laboratory. Three aliquots of 100 µl each of the rinse suspensions and appropriate dilutions were filtered through 0.6 µm filters (Schleicher & Schuell) on Tryptose Blood Agar (TBA) (Oxoid) plates added with 10% of laked horse blood (Oxoid). The filters were removed after 30 minutes and the plates were incubated for 2-5 days at 37°C into 80% N₂, 10% CO₂ and 10% H₂ obtained by flushing the gas mixture through a jar previously evacuated.

In order to test the possibility to isolate the emerging Campylobacter species under study using the Cape Town method, one set of rinse water samples was challenged with 10⁷ CFU/ml of *C. upsaliensis* CCUG 19559, *C. helveticus* CCUG 34016, *C. hyointestinalis* LMG 7530 and *C. lari* CCUG 22395. All the challenged samples were processed as previously described.

At the end of the incubation period, up to five Campylobacter-like colonies per positive sample were purified onto TBA plates added with 10% of laked horse blood, submitted to chromosomal DNA extraction (Josefsen et al., 2004) and identified by using two PCR assays designed for the identification of *C. jejuni*, *C. coli* and Campylobacter spp. (Manfreda et al., 2003) and *C. upsaliensis*, *C. helveticus*, *C. hyointestinalis*, *C. lari* (Linton et al., 1996), respectively.

A total of 243 poultry meat based foods, represented by broiler carcasses (N=86), chicken wings (N=86) and chicken legs (N=86), were tested for the presence of emerging Campylobacter species by using the CAMPYCHECK protocol. All meat products were collected from February 2005 to January 2006 in the slaughterhouse after packaging or at retail, before the expiration date, in three regions located in the north of Italy named Emilia Romagna, Lombardia and Veneto. In these regions it is concentrated more than 50% of the Italian chicken meat production.

An aliquot of 25 gr of each sample was placed in stomacher bags containing 225 ml of Campylobacter Enrichment broth (CEB) (Oxoid) supplemented with 5% laked horse blood and pulsified for 15 sec before incubation at 37°C in the CAMPYCHECK Gas Pack (Oxoid). At the end of the incubation period, 10 ml of the enrichment broth were centrifuged at 2500 rpm for 10 sec and 1 ml of the supernatant was diluted in 9 ml of Maximum Recovery Diluent (Oxoid). Then, 200 µl of the neat enrichment broth and of its dilution 1:10 were filtered through 0.6 µm filters on Anaerobic Basal Agar (ABA) (Oxoid) plates added with 5% of laked horse blood. The filters were removed after 30 minutes and the inoculum spread into the plates then incubated for 2-5 days at 37°C in the CAMPYCHECK Gas Pack.

One to four Campylobacter-like colonies were selected from each positive sample, purified onto ABA plates added with 5% of laked horse blood, analysed under the microscope and then identified using the PCR protocols previously described.

The presence of Campylobacter positive samples was correlated to the meat sampling season. Based upon varying anticipated temperatures during each sampling, September and October were designated as autumn, November through February as winter, March and April as spring, and May through August as summer.

The data collected were analysed with the Statgraphics® package (ver. 5.1) (StatSoft, Inc.). The number of positive samples detected among meat samples were compared by using the Scheffe test and P ≤ 0.05 was considered statistically significant.
Results and discussion

The Campylobacter prevalence on broiler carcasses estimated by using the Cape Town protocol was 100%. In fact, all 222 broiler carcasses tested were Campylobacter positive. A total of 656 Campylobacter colonies were isolated from 145 of the 222 positive carcasses. According to the mPCR results, all these isolates were classified as thermophilic species. In particular, 48.6% as \textit{C. coli} and 51.4% as \textit{C. jejuni}. An example of PCR gel in which \textit{C. coli} and \textit{C. jejuni} were identified is showed in Figure 1. The only samples in which were isolated colonies then identified as emerging species were those experimentally inoculated.

![Example of thermophilic species identification by mPCR](image)

The Campylobacter prevalence detected among poultry meat samples was 46.1%. In fact, 112 over 243 chicken meat samples tested using the CAMPYCHECK protocol were Campylobacter positive. The number of positive samples among broiler carcasses (N=35), chicken wings (N=39) and chicken legs (N=38) did not show any statistically significative difference. On the contrary the percentages of positive chicken meat samples detected in spring and summer were significantly higher that that detected winter (Figure 2).

![Number of Campylobacter meat samples in relation to the sampling seasons](image)

The CAMPYCHECK gas pack, in which the meat samples were incubated, was set up during the CAMPYCHECK project. This gas pack has been described by Smith et al. (2006) and include the...
addition of a CampyGen, a AnaeroGen and a CO\textsubscript{2}Gen sachets, with a supplement of 0.1g of sodium borohydrate in 10 ml of water, into a 3.5 litre jar.

In this study the application of a gas incubation atmosphere containing hydrogen, an incubation temperature of 37 instead of 42\textdegree{}C and the use of filtration onto blood agar instead of selective agars did not allowed to isolate Campylobacters belonging to the species \textit{C. upsaliensis}, \textit{C. helveticus}, \textit{C. hyointestinalis} and \textit{C. lari}. Further studies will be performed in order to evaluate if these species do not colonies poultry at all or if they are present onto broiler carcasses and poultry meat at low concentration and thermophilic species compete with them inhibiting their possibility to show up on plates. The application of an enrichment step in the CAMPYCHECK protocol should improve the cell viability and their possibility to move through the filters but it is not selective for emerging species and it allows the growth of thermophilic Campylobacters even in presence of hydrogen at 37\textdegree{}C.

References


