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Prevalence and characterization of *Campylobacter jejuni* from chicken meat sold in French retail outlets

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ABSTRACT

Campylobacter was detected in 76% of broiler meat products collected in retail outlets during a monitoring plan carried out in France throughout 2009. *Campylobacter jejuni* was the most prevalent species (64.7% of products being contaminated). The 175 *C. jejuni* isolates collected were characterized. MLST typing results confirmed substantial genetic diversity as the 175 *C. jejuni* isolates generated 76 sequence types (STs). The ST-21, ST-45 and ST-464 complexes predominated accounting for 43% of all isolates.

A class-specific PCR to screen the sialylated lipooligosaccharide (LOS) locus classes A, B and C showed that 50.3% of the *C. jejuni* isolates harbored sialylated LOS.

The antimicrobial resistance profiles established using a subset of 97 isolates showed that resistance to tetracycline was the most common (53.6%), followed with ciprofloxacin and nalidixic acid (32.9%, and 32.0% respectively). All the tested isolates were susceptible to erythromycin, chloramphenicol and gentamicin.

Clear associations were demonstrated between certain clonal complexes and LOS locus classes and between certain clonal complexes and antimicrobial resistance.

This work paints a representative picture of *C. jejuni* isolated from poultry products circulating in France, providing data on STs, LOS locus classes and antibiotic resistance profiles in isolates recovered from products directly available to the consumer.

1. Introduction

Campylobacter jejuni is the major cause of bacterial gastroenteritis in developed countries. Over 214,000 human cases were reported in 2012 in the European Union (EFSA and ECDC, 2014). Broiler chickens are generally recognized as the major source of contamination and the handling, preparation and consumption of broiler meat may account for 20 to 30% of human campylobacteriosis cases (EFSA, 2010b).

Campylobacteriosis is usually a self-limiting disease lasting only a few days. Treatment with antimicrobials is not usually required except in severe cases or immunocompromised patients. Macrolides, such as erythromycin are now generally considered to be the optimal drug for treating campylobacteriosis, with fluoroquinolones as an alternative therapy (Allos, 2001). However, the prevalence of antimicrobial resistant *Campylobacter* strains in humans is increasing especially resistance to fluoroquinolones and to a lesser extent, macrolides (Cody et al., 2010; EFSA and ECDC, 2011). Multiple resistance patterns to several classes of antibiotics are also emerging. Trends in microbial resistance have shown a clear link between use of antibiotics in the poultry industry and resistant isolates of *Campylobacter* in humans (Moore et al., 2006). These resistant zoonotic bacteria are of special concern since they might compromise the effective treatment of infections in humans.

C. jejuni is also a recognized antecedent cause of post-infectious neuropathies such as Guillain–Barré syndrome (GBS) or Miller–Fischer syndrome (MFS) (Nachamkin et al., 1998). Among the numerous factors involved in the development of such autoimmune diseases, molecular mimicry between the lipooligosaccharide (LOS) structures present on the cell surface of *C. jejuni* and gangliosides in peripheral nerves play a crucial role (Ang et al., 2004; Ang et al., 2002; Godschalk et al., 2004).

Nineteen distinct classes of LOS biosynthesis loci have been described based on major genetic differences, gene content and

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organization (Parker et al., 2008). Five of these classes (A, B, C, M and R) harbor sialyltransferase genes involved in incorporating sialic acids into the LOS (Gilbert et al., 2002; Parker et al., 2008; Parker et al., 2005). Sialylation of the LOS core has been associated with ganglioside mimicry and it has been shown that in GBS or MFS related to *Campylobacter* strains, the majority of strains express the LOS gene cluster A or B (Godschalk et al., 2004; Islam et al., 2009; Koga et al., 2006).

Moreover, *C. jejuni* expressing sialylated LOS (classes A, B and C) appear to be more invasive for intestinal cells *in vitro* than those expressing non-sialylated LOS (classes D and E) (Habib et al., 2009a; Louwen et al., 2008). Sialylation of the LOS was also associated with increased severity of enteric disease by Mortensen et al. (2009) but these results were not recently supported by Ellström et al. (2013).

Molecular typing is an important tool for elucidating the diversity and transmission routes of *Campylobacter* isolates contaminating the food chain. Various molecular typing methods are currently available for studying the population structure of *Campylobacter* (Wassenaar and Newell, 2000). These genotypic methods have shown that *Campylobacter* has marked genomic diversity and a slightly clonal population structure, in which the different lineages and the relatedness between isolates cannot be easily determined, particularly within the framework of long-term epidemiological studies. Based on a concept similar to multilocus enzyme electrophoresis, multilocus sequence typing (MLST) exploits the relative conservation in sequence of certain genes in which variations are more likely to be selectively neutral because of their housekeeping functions (Maiden et al., 1998). This approach was successfully developed to study *Campylobacter* populations (Dingle et al., 2001) and is now recognized as the gold standard typing method for this bacterial genus (Sheppard et al., 2009).

In France, little is known about the prevalence of pathogenic *Campylobacter* in chicken and their molecular characteristics. No population-based surveys have been conducted to investigate the molecular epidemiology of *C. jejuni* in chicken meat at points close to human consumption. In this study, a survey was carried out to investigate the prevalence of *Campylobacter* in the chicken meat supply chain. *C. jejuni* isolates were further characterized as in France, more than 80% of campylobacteriosis are due to this species. Thus MLST was used to determine the diversity and clonal relationships among the 175 *C. jejuni* isolates collected. In addition, we correlated the LOS classes assigned by PCR and antimicrobial resistance of the isolates with the genotypes assigned by MLST.

2. Material and methods

2.1. Sampling of the monitoring plan

The monitoring plan concerned three types of chicken meat products: 120 carcasses, 121 chicken legs and 120 chicken fillets collected in retail outlets from April to December 2009. The plan was carried out in geographic areas representing the most significant consumption patterns in France (Anonymous, 2010). Chicken meat products were analyzed at the ANSES Laboratory of Ploufragan–Plouzané which is the French National Reference Laboratory for *Campylobacter*.

2.2. Bacteriological analysis

Enumeration and sample enrichment were both used to recover *Campylobacter* from the chicken products. Twenty eight grams of skin from the wishbone and neck (from carcasses), 28 g of skin (from chicken legs) or 28 g of muscle (from fillets) was put into a sterile bag and diluted using a ratio of 1:10 in a tryptone salt broth. The mixture was then homogenized for 1 min in a peristaltic homogenizer. Detection by enrichment and enumeration procedures was performed according to parts 1 and 2 respectively of the NF EN ISO 10272 standard (Anonymous, 2006). For detection purposes, 10 mL of the homogenate was added to 90 mL of Bolton broth (Oxoid, Dardilly, France). The inoculated broth was then incubated under microaerobic conditions for 4 h at 37 °C and then for 44 ± 4 h at 41.5 ± 1 °C. Subsequently, 10 μL of the culture was plated onto mCCDA (modified Charcoal Cefoperazone Deoxycholate Agar) and Butzler agar (Virion N°2) (Oxoid, Dardilly, France) and incubated for 44 ± 4 h at 41.5 ± 1 °C. For each positive plate, up to two typical *Campylobacter* colonies were subcultured onto blood agar plates for further characterization according to the NF EN ISO 10272-1 standard (Anonymous, 2006). Then the two isolates were analyzed for species determination by PCR (Denis et al., 1999). *Campylobacter* was enumerated by duplicate plating 1 mL of the homogenate onto three mCCDA plates. Tenfold serial dilutions of the homogenate in tryptone salt broth were also prepared and plated onto one mCCDA plate. All the plates were incubated under microaerobic conditions for 44 ± 4 h at 41.5 ± 1 °C. The enumeration limit was 5 CFU/g. Only *C. jejuni* isolates were further characterized in this study with one isolate per sample. The isolate collection was stored at −70 °C in peptone broth containing 20% (v/v) glycerol. When required, isolates were obtained from frozen stock after plating on Karmali agar (Oxoid, Dardilly, France) for 48 h under a microaerophilic atmosphere.

2.3. MLST

DNA was prepared using the InstaGene® Matrix (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's recommendations. The seven housekeeping genes for MLST (*asp*A, *gln*A, *glt*A, *gly*A, *pgm*, *tkt* and *unc*A) were amplified and sequenced according to previously developed experimental conditions (Dingle et al., 2001). PCR products were cleaned up using the ExoSAP-IT treatment (GE Healthcare), and sequence extension reactions carried out in BigDye Ready reaction mix according to the manufacturer's instructions. Unincorporated dye terminators were removed by an ethanol precipitation method before the products were analyzed with an ABI Prism 3130 sequencer (Applied Biosystems). The sequences were assembled using the assembler implemented in BioNumerics v. 6.5 software. All allelic sequences were queried against the *C. jejuni* MLST database (http://pubmlst.org/campylobacter). Alleles already present in the database were assigned the numbers given there; novel alleles and sequence types (STs) were submitted to the *C. jejuni* MLST database and assigned new numbers. Sequence types were assigned into genetically related clusters called clonal complexes (CCs), based on sharing four or more alleles with the central genotype that had been identified in previous studies using the BURST algorithm and UPGMA cluster analysis (Dingle et al., 2002).

2.4. Determination of the LOS locus class by PCR

Primer sets used for the LOS locus class determination are presented in Table 1. The primer set for the *cstII* gene gave amplification for both LOS class A and LOS class B isolates. LOS class A isolates were then distinguished from class B isolates because they were not amplified with the class B-specific primers (*cgta-II*b). Assays were performed using a MyCycler® thermocycler (BioRad Laboratories, Hercules, CA, USA) with a program consisting of an initial denaturation step of 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C and a final extension of 7 min at 72 °C. PCR reactions were carried out in a 25 μL volume containing 1 μL DNA template, 1× buffer, 200 μM each of deoxynucleoside triphosphate (Clontech Laboratories, Mountain View, CA, USA), 0.5 μM of each PCR primer, 3 mM $MgCl_2$ and 0.6 U of *Taq* polymerase (AmpliTaq®, Applied Biosystems, Foster City, CA, USA). DNA from strains with a known LOS class was used as controls: strain C66 isolated in the laboratory as a class A reference strain, *C. jejuni* 81–176, as a class B, *C. jejuni* NCTC11168 as a class C and *C. jejuni* 81116 for other classes.