Phenotypes and genotypes of campylobacter strains isolated after cleaning and disinfection in poultry slaughterhouses.

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Accepted Manuscript

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PII: S0378-1135(07)00524-X
DOI: doi:10.1016/j.vetmic.2007.10.021
Reference: VETMIC 3860

To appear in: *VETMIC*

Received date: 18-7-2007
Revised date: 16-10-2007
Accepted date: 17-10-2007

Please cite this article as: Peyrat, M.B., Soumet, C., Maris, P., Sanders, P., Phenotypes and genotypes of campylobacter strains isolated after cleaning and disinfection in poultry slaughterhouses, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.10.021

1 **Phenotypes and genotypes of campylobacter strains isolated after cleaning and disinfection in**
2 **poultry slaughterhouses**

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Abstract

Campylobacter is responsible for human bacterial enteritis and poultry meat is recognised as a primary source of infection. In slaughterhouses, cleaning and disinfection procedures are performed daily, and it has been suggested that disinfectant molecules might select for antibiotic resistant strains if shared targets or combined resistance mechanisms were involved. The aim of the study was to investigate if cleaning and disinfection procedures in poultry slaughterhouses select for antibiotic resistance in *Campylobacter jejuni* and *C. coli* and to determine the genotypes of isolates collected after cleaning and disinfection. Nine sampling visits were made to four French slaughterhouses. Samples were collected from transport crates and equipment surfaces, before and after cleaning and disinfection. Minimal inhibitory concentrations of the recovered *C. jejuni* and *C. coli* isolates to 6 antibiotics and 2 disinfectants were measured. The *C. jejuni* isolates collected from equipment surfaces after cleaning and disinfection were subjected to PCR-RFLP typing. Twenty-five *C. jejuni* isolates and 1 *C. coli* were recovered from equipment surfaces after cleaning and disinfection during 5 visits to 3 different slaughterhouses. Those isolates didn't show an increased resistance to the tested antibiotics compared to isolates collected before cleaning and disinfection. Only one or two genotypes were recovered after cleaning and disinfection during single visits to each slaughterhouse. This observation suggests that such genotypes may be particularly adapted to survive cleaning and disinfection stress. Understanding the survival mechanisms of *Campylobacter* should facilitate the implementation of better targeted strategies and reduce the public health burden associated with *Campylobacter* infection.

Keywords: campylobacter; slaughterhouse, disinfectant, antibiotic, cross-resistance, PCR-RFLP genotyping

1. Introduction

Campylobacter is one of the most common causes of human bacterial enteritis worldwide. Thermophilic campylobacters, in particular *Campylobacter jejuni* and its close relative *C. coli,* are the predominant cause of campylobacter infections (Anonymous, 2003). There is significant

epidemiological evidence to suggest that poultry meat is a primary source of human campylobacter infection (Anonymous, 2003). *C. jejuni* and *C. coli* are generally considered to exist commensally in the gastro-intestinal tract of birds, particularly poultry. In slaughterhouses, carcass contamination with spilled gut contents occurs during evisceration (Borck and Pedersen, 2005).

Campylobacter are highly sensitive to environmental stress. One of the most intriguing aspects of campylobacter research is the apparent sensitivity of the microorganism under laboratory conditions and its contrasting persistence in the food chain and the incidence of human infection (Park, 2005). Cleaning and disinfection procedures in slaughterhouses are performed daily in each sector of the slaughter process. In this article, the expressions “cleaning and disinfection” will be shortened to “cleaning”. Transport crates are usually cleaned and disinfected between each use. Although Campylobacter is generally considered sensitive to disinfectants (Avrain et al., 2003; Blaser et al., 1986; Trachoo and Frank, 2002; Wang et al., 1983), it can be routinely detected in floor surface swabs of commercial transport cages after cleaning (Newell et al., 2001; Slader et al., 2002). It has been speculated that the increasing use of chemical disinfection, particularly of quaternary ammonium compounds, might impose a selective pressure and contribute to the emergence of disinfectant-resistant microorganisms (Langsrud et al., 2003) and that biocides might select for antibiotic resistant strains (Russell, 2000). The emergence of microorganisms exhibiting combined resistance to disinfectants and antibiotics represents a public health burden.

The ability of a bacterium to survive the variety of stresses experienced during cleaning procedures is dependent on the presence and expression of stress response genes. Such properties might affect the genetic diversity of campylobacter population before and after cleaning. Different genotyping methods have been used to characterise *C. jejuni* and restriction fragment length polymorphism (RFLP) of the *fla*A polymerase chain reaction (PCR) product appears to be a valuable method for epidemiological investigations (Wassenaar and Newell, 2000).

These observations led us to hypothesize that the campylobacter strains isolated after cleaning procedures might present a higher resistance to antibiotics and have a specific genotype. We therefore measured and compared the minimal inhibitory concentrations to antibiotics and disinfectants of isolates collected before and after cleaning and determined the genotypes of the *C. jejuni* isolates collected after cleaning.

2. Materials and method

2.1. Collection of samples in four poultry slaughterhouses

Four unrelated French poultry slaughterhouses (designated A, B, C and D located in Brittany and Pays de la Loire) were visited from August 2005 to June 2006. Each plant processed industrial poultry to finished products such as carcasses and pieces with capacities of 6000 to 9000 birds per hour for broilers, 5000 guinea fowls per hour and 2000 turkeys or ducks per hour. Plants A and C were investigated once, plant B three times, and plant D four times. Plants B and C processed chickens, turkeys and guinea fowl on the same chain. Plant D processed poultry and guinea fowl on one chain and turkeys and ducks on another. Plant A only processed turkeys. The slaughter chains for broilers and guinea fowl were entirely automated. In turkeys and ducks, most of the evisceration chain after plucking was carried out manually.

2.2. Audit of cleaning and disinfection procedures in slaughterhouses

A questionnaire was sent to the quality control manager of each of the 4 slaughterhouses. The objective was to know which families of disinfectant molecules were used in different sectors. We focused on the disinfectant molecules used to disinfect the transport crates and the equipment surfaces in contact with poultry carcasses during processing.

2.3. Sampling of transport crates

Samples were taken from the transport crates of the flocks slaughtered in each slaughterhouse. A flock was defined as all birds reared in the same poultry house and slaughtered on the same day. Samples were taken from 5 different transport crates for each flock. At least 10 fresh droppings were taken and pooled before cleaning. Sterile gauze swabs (10x10 cm) soaked in sterile saline were used to collect samples *ad random* from the transport crates (sides and bottom) after cleaning. Swabs were wiped vigorously over the bottom and door of the cages and placed in sterile stomacher bags. All

samples were kept at 4°C until further processing within 48 h. The transport crates of 43 poultry flocks were sampled.

2.4. Sampling of equipment and scald tank water

As cleaning was carried out at the end of the working day, the surfaces and scald tank water were sampled on two consecutive days: the first day, at the end of processing, before cleaning, and the next day, before starting the slaughter process, after cleaning (one of the 3 visits to slaughterhouse B occurred only after cleaning). A total of 101 environmental swabs were collected, 45 before and 56 after cleaning. Sterile gauze swabs (10x10 cm) soaked in sterile saline were used to collect samples from the processing equipment, which included the rubber fingers of the defeathering machine, the evisceration machines and the conveyor belts. The swabs were wiped vigorously over the appropriate area for approximately 30 s and placed in sterile stomacher bags. The size of the area depended on the type of surface. At each visit, 250 ml of scald tank water were collected before and after cleaning of the scald tank. All samples were kept at 4°C until further processing within 48 h.

2.5. Isolation and identification of Campylobacter spp.

Campylobacter detection was carried out according to the standard French method (AFNOR, 1996). All samples were subjected to a selective enrichment step before bacterial isolation. The *Campylobacter* isolates were cultured in a microaerophilic atmosphere (5% O_2, 10% CO_2, 85% N_2) at 42°C.

Ten g of each sample of pooled droppings were aseptically weighed and placed in sterile stomacher filter bags with 90 ml of Preston broth consisting of nutrient broth N° 2 (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) defibrinated sheep blood (AES laboratories, Combourg, France) and campylobacter-selective Preston supplement (SR0117E, Oxoid). The samples from the scald water tank were centrifuged at 3000 g for 20 min and the pellet resuspended in 100 ml of Preston broth. Swabs were placed directly in 100 ml of Preston broth. Each bag was blended for 30 s prior to incubation. After 24 h, 10 µl of enrichment broth were plated onto Karmali agar (*Campylobacter* agar base (CM935) supplemented with the Campylobater-selective Karmali supplements SR0167E, Oxoid)

and Virion media (Goossens et al., 1983) and incubated under microaerophilic conditions for 48 h. Two characteristic colonies from each plate were transferred onto blood agar medium (Mueller Hinton agar base (Difco, Becton Dickinson, le Pont de Claix, France) with 5% (v/v) defibrinated sheep blood, AES Laboratories) and incubated for 48 h.

The species of each isolate was identified using a method previously developed by Denis *et al.* (2001) with some modifications. Briefly, isolated colonies were picked from the agar plate and suspended in 1 ml of distilled water in a microfuge tube. Samples were boiled for 10 min at 95°C and cooled on ice before being added to the PCR mix. Specific primers selected from *mapA* gene and *ceuE* genes were used to simultaneously detect the species *C. jejuni* and *C. coli* (Table 1). The PCR reactions were performed using a Gene Amp 9700 thermocycler (Perkin Elmer Instruments, Norwalk CT, USA) in 30 µl of mixture containing 1 U *AmpliTaq* Gold (Applied Biosystems), 100 µmol/l each of deoxynucleoside triphosphate, 1X GeneAmp Buffer with 2.5 mmol/l $MgCl_2$, 0.42 µmol/l each of MdmapA1, MdmapA2, Mdcol2 and Mdcol3 primers and 5 µl of boiled cell suspension as template. The reaction included an initial denaturation of DNA at 94°C for 7 min and then 35 consecutive cycles of denaturation (30 s, 94°C), primer annealing (30 s, 52°C), and extension (72°C, 30 s). A final elongation step was performed for 10 min at 72°C. PCR products (10 µl) were separated by electrophoresis for 1 h 30 at 110 V on 1% agarose gel (agarose standard, Eurobio, France) stained with ethidium bromide (0.5 µg/ml) and viewed under UV light. A 589 bp PCR product was obtained for *C. jejuni* and 462 bp for *C. coli*. For each positive PCR result, colonies were transferred to a peptone broth with 15% of glycerol and frozen at –80°C before phenotype and genotype analysis.

2.6. Antimicrobial agents and antimicrobial susceptibility testing

The antibiotics tested were: ampicillin, tetracycline, gentamicin, streptomycin, erythromycin, and enrofloxacin purchased from Sigma Aldrich (St Quentin-Fallavier, France). The disinfectants were benzalkonium chloride (BTC50®; Stepan Europe, Voreppe, France) and didecyl-dimethyl ammonium chloride (Bardac 22®, Lonza, Basel, Switzerland).

The minimum inhibitory concentrations (MIC) of the antimicrobial agents were determined on Mueller-Hinton agar (Difco) supplemented with 5% defibrinated sheep blood by an agar dilution method, in

accordance with the CLSI formerly NCCLS document M7-A6 (NCCLS, 2003). Cultures were grown on Karmali agar plates for 48 h under microaerophilic conditions at 37°C.

Antibiotic susceptibility was determined according to guideline 2007 of the "Comité de l'antibiogramme de la société française de microbiologie" (http://www.sfm.asso.fr last access: 11/05/2007). The antibiotic and disinfectant molecules, their respective concentrations (in two-fold increases) and resistance breakpoints are shown in table 2. *Campylobacter jejuni* ATCC33560 and *Campylobacter coli* ATCC33559 were used as quality controls (NCCLS document M31-A2) (NCCLS, 2002) for each MIC determination. Each measure was repeated twice. The choice of antibiotics was made in accordance with the national antimicrobial resistance monitoring system (Anonymous, 2006). Quaternary ammonium compounds were selected as disinfectants as these were used in the slaughterhouses visited.

2.7. Genotyping of Campylobacter spp: PCR-RFLP of the pfla/gyrA and flaA genes

All *C. jejuni* isolates from samples collected after cleaning of slaughterhouse surfaces were genotyped. *Campylobacter* isolates were incubated on Karmali agar for 48 h at 37°C under microaerophilic conditions. Cells were harvested and resuspended in 1.5 ml of Brucella broth. Total DNA was extracted using the Nucleospin® Tissue kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's instructions. DNA samples were kept at –20°C for further analysis.

PCR was performed in a 50 µl reaction volume with a Chromo-4 thermal cycler (Biorad S.A., Marnes la coquette, France) according to the conditions described by Ragimbeau *et al.* (1998) with slight modifications. The reaction mixture consisted of 1X XL PCR buffer II (Applied Biosystems, Courtaboeuf, France) with 1 mmol/l $Mg(Oac)_2$, 1.5 U *rTth* polymerase XL (Applied Biosystems), 0.4 µmol/l of each primer, 0.2 mmol/l concentrations of deoxynucleoside triphosphates (Applied Biosystems) and 5 µl of template DNA at 10 pmol/l. The reaction included an initial denaturation of DNA at 94°C for 1 min and then 35 consecutive cycles of denaturation (15 s, 94°C), primer annealing (30 s, 45°C) and chain extension with a ramp of 2 min to 68°C. A final extension step was performed for 10 min at 72°C. The amplified product size was 1448 bp for the *fla*A gene (Table 1).

Amplification of *pflA* and *gyrA* genes by multiplex PCR was carried out as previously described by Ragimbeau *et al.* (1998) (Table1).

The presence of the expected PCR product was verified by subjecting 5 µl of the reaction mixture to electrophoresis on a 1 % agarose gel (electrophoresis grade agarose, InVitrogen, Cergy Pontoise, France) for 90 min at 110 V and stained with ethidium bromide solution (1µg/ml). Amplified products were visualized under UV light.

For *fla*A gene polymorphism, 8 µl of PCR products were digested in 30 µl with 10 U of *DdeI* (Qbiogen, Illkirch, France) in buffer number 3 added to 100 ng/µl bovine serum albumin (BSA, Q Biogen). For pfla/gyrA polymorphism, 15 µl of PCR products were simultaneously digested with 1 U of *HindIII, HinfI, HhaI* and *DdeI* (Q Biogen) in a total volume of 30 µl with buffer number 2 added to BSA.

The reaction mixture was incubated in a water bath for 4 h at 37°C. The digested PCR products (10 µl) were analysed by electrophoresis at 3 V/cm for 4 h on 2.5 % ethidium bromide (2 µg/ml) stained agarose gel in 1X TBE (Tris 89 mmol/l; borate 89 mmol/l, EDTA 2 mmol/l pH 8.3). The molecular size markers were ΦX174-*HaeIII* (Promega, Charbonnières les bains, France) and 100 bp DNA ladder (Promega). The restriction enzyme profiles were visualized under UV light and images were captured by Bio 1-D analyser (Fisher Bioblock Scientific, Illkirch, France). The electrophoretic profiles were compared visually.

2.8. Statistical analysis

Statistical analyses were performed with Systat 9® for Windows (Systat, Inc., 1800 Sherman Ave., Evanston, Illinois, USA). The isolation percentages and MIC distributions were compared by Chi^2 or exact Fisher test. Results were considered significant when $p \leq 0.05$. A Spearman coefficient was calculated for the correlation matrix between MIC distributions.

3. Results

3.1 Cleaning and disinfection procedures used in slaughterhouses

Three of the four slaughterhouses visited (A, B and D) completed our questionnaire about cleaning and disinfection procedures.

The transport crates were cleaned with cold water (10-15°C). In slaughterhouse D, the transport crates were turned in a tunnel during cleaning, and large pieces of dirt (feathers, droppings or chicken legs) could be removed from the crate. In the other slaughterhouses, the cleaning system did not allow reversal of the crates, and large pieces of dirt were still present at the end of the cleaning procedure. The transport crates were disinfected with a mixture of quaternary ammonium and glutaraldehyde in slaughterhouses A and D, and with chlorine compounds in slaughterhouse B. Organic matter was still visible in the transport crates at the end of the cleaning procedure in all slaughterhouses visited. The slaughterhouse equipment was pre-cleaned with high-pressure water. It was then cleaned using a device with foam containing alkaline – chlorinated molecules in slaughterhouses A and D and with a neutral detergent in slaughterhouse B. Quaternary ammonium compounds combined with glutaraldehyde were used to disinfect equipment in slaughterhouses A and B. Equipment in slaughterhouse D was disinfected with a formulation containing poly (hexamethylene biguanide) hydrochloride. Disinfectant product, concentration and contact times are indicated on table 3.

3.2. Isolation percentages before and after cleaning and disinfection

Campylobacter was isolated from 81% (35/43) of the crate samples before cleaning and in 77% (33/43) after cleaning (data not shown). Although different methods and molecules were used by the slaughterhouses to clean and disinfect their transport crates, no significant difference between the different slaughterhouses was observed for the percentages of campylobacter isolation from transport crates before and after cleaning procedures ($p > 0.05$). Different types of transport crates are used to transport poultry: those for turkeys are made with metal and those for chickens in plastic. No significant difference in campylobacter isolation percentage was observed between the two types of transport crate (data not shown, $p > 0.05$). *C. jejuni* was isolated from 28, and *C. coli* from 12 of the 35 positive samples before cleaning, and in 28 and 9 respectively of the 33 positive samples after cleaning. No significant difference between the isolation percentages of the two campylobacter species from transport crates was observed before or after cleaning procedures (($p > 0.05$) data not shown).

Results of sampling from slaughterhouse equipment are shown in table 4. Campylobacter was recovered in all slaughterhouses before cleaning and in 3 of the 4 slaughterhouses after cleaning.

Campylobacter was isolated from 80 % (36/45) of surface sample swabs taken before cleaning and from 18 % (10/56) of swabs collected after cleaning. No difference was observed between the different positive slaughterhouses nor the different sample sites (data not shown) ($p > 0.05$).

Both species could be isolated from the same sample. We isolated *C. jejuni* from 29 of the 36 positive samples, and *C. coli* from 10 samples before cleaning, and from 9 and 1 respectively of the 10 positive samples after cleaning. There was no statistically significant difference between species isolation percentages on equipment surfaces before and after cleaning ($p > 0.05$).

Campylobacter was detected in 71% (5/7) of water samples taken before cleaning and in none of the water samples collected after cleaning of the scald tank.

3.3. Susceptibility to antibiotics before and after cleaning and disinfection

In transport crates, 142 *C. jejuni* isolates and 44 *C. coli* isolates were collected. The antibiotics and disinfectant MICs of all isolates collected from transport crate samples were measured. None of the isolates of *C. jejuni* and *C. coli* from transport crate samples was resistant to gentamicin and none of the *C. jejuni* isolates was resistant to streptomycin (data not shown). All isolates of *C. coli* collected from transport crates were resistant to tetracycline. No statistically significant difference was observed between resistance percentage to antibiotics before and after cleaning of transport crates.

An isolate is considered multidrug resistant when it is resistant to more than one antibiotic. No statistically significant differences in multidrug resistant percentages before and after cleaning were observed for either *C. jejuni* or *C. coli* from transport crates (data not shown, $p > 0.05$).

From surface samples of slaughterhouse equipment, 135 isolates were collected, 85 % (115/135) were C. *jejuni* and 15 % (20/135) C. *coli* (Table 4). As shown in Table 4, only one isolate of *C. coli* was collected after cleaning, so the resistance percentages before and after cleaning were compared for *C. jejuni* only. No *C. jejuni* isolates from swabs of slaughterhouse equipment were resistant to gentamicin. The histogram of antibiotic resistant percentages before and after cleaning is shown in Figure 1. No statistically significant difference between resistances to tetracycline, erythromycin, enrofloxacin and streptomycin ($p > 0.05$) were observed before and after cleaning. A significant difference was observed for ampicillin, (Fisher exact test: $p = 0.042$). Isolates obtained after cleaning

were less resistant to ampicillin than isolates obtained before cleaning. The overall results in Figure 1 show a slight decrease in the level of antibiotic resistance.

A complete antibiotic resistance pattern was obtained for only 96 *C. jejuni* isolates. The antibiotic resistance profiles of *C. jejuni* isolates collected before and after cleaning procedures are shown in Table 5. We observed 9 phenotypes before cleaning and 5 after, but differences between the profile distributions before and after cleaning were not significant (Chi² test, $p > 0.05$).

A statistically significant difference was observed between the multidrug resistant percentage before and after cleaning of the equipment surfaces. *C. jejuni* isolates collected after cleaning were less multidrug resistant than those collected before cleaning (Chi² test, $p = 0.011$)

3.4. Disinfectant susceptibility before and after cleaning and disinfection

The MIC distributions for benzalkonium chloride and didecyl-dimethyl-ammonium chloride were examined for isolates from transport crates and equipment surfaces before and after cleaning. Figure 2 shows the histograms of isolates collected before and after cleaning from equipment surfaces. The MIC distributions of the disinfectant are monomodal and limited to only a few values. No difference between the MIC distributions before and after cleaning was observed for transport crates and equipment surfaces ($p > 0.05$).

3.5. Correlation between the distributions of antibiotic MICs and disinfectant MICs

A Spearman correlation matrix was calculated for the 6 distributions of antibiotic MICs and the 2 distributions of disinfectant MICs. A correlation between MICs distribution of gentamicin and streptomycin (antibiotics of the aminoglycoside family) was observed for both species ($r = 0.479$ for *C. jejuni* and 0. 571 for *C. coli*). Another correlation was observed for both species between quaternary ammonium molecules ($r = 0.684$ for *C. jejuni* and 0.514 for *C. coli*). No correlation was observed between antibiotic and disinfectant MICs.

3.6. Genotypes of isolates collected after cleaning and disinfection

Twenty-five *C. jejuni* isolates collected after cleaning were genotyped. Profiles of *pfl*A/*gyr*A and *fla*A migrations are shown in figure 3. Eight different genotypes were apparent from *pfl*A/*gyr*A (A, B, C, D, E or F) and *fla*A profiles (a, b, c, d, e, f, g and h). Most genotypes (6/8) were recovered from only one sampling location. One genotype (Bb) was recovered from 4 different sampling sites, and one (De) from 3 different sampling sites. Conversely, two genotypes (Bb and Cc) were observed at the same site (Table 6). No isolate with the same genotype was ever recovered during successive visits to the same slaughterhouse. One to 10 *C. jejuni* isolates were collected from each slaughterhouse after cleaning, (mean=5). No more than two genotypes were observed during any slaughterhouse visit after cleaning.

4. Discussion

Campylobacter are the most fastidious and stress-sensitive of the common food-borne pathogens (Park, 2002). However their mechanisms of survival are such that they can survive in the slaughterhouse environment and also survive cleaning procedures.

Different methods and disinfectant molecules were used to clean and disinfect the transport crates in the four slaughterhouses visited, with or without reversal of the crates. However, organic matter was still regularly detected on the washed crates in all slaughterhouses. This has already been reported in other studies (Berrang and Northcutt, 2005; Slader et al., 2002). Disinfection had no effect on the percentage of campylobacter isolation from transport crates in our study. Other investigations have revealed that the cleaning process had little (if any) effect on the campylobacter status of transport crates (Slader et al., 2002). This observation is of concern for public health as it has also been demonstrated that transport crates can be a source of campylobacter-free flock contamination by campylobacter (Newell et al., 2001). None of the treatments eliminated campylobacter as the organic matter protected bacteria from contact with disinfectant molecules and decreased the efficiency of these molecules.

The equipment in the four slaughterhouses visited was cleaned and disinfected by an outside company. The procedures were relatively standardized, starting in all cases with removal of the organic matter with high-pressure water, then application of detergent and disinfectant molecules to

surfaces presumed free of organic matter. Although little information is available about campylobacter susceptibility to disinfectant, this pathogen is generally considered susceptible to the disinfectants used in the food industry, especially quaternary ammonium and chlorine compounds (Avrain et al., 2003; Blaser et al., 1986; Wang et al., 1983). To our knowledge, this is the first description of campylobacter isolates collected from equipment surfaces in poultry slaughterhouses after cleaning. A few studies have been carried out to detect campylobacter after cleaning in the food industry environment (Borck and Pedersen, 2005; Cools et al., 2005; Malakauskas et al., 2006; Miwa et al., 2003). All samples collected after cleaning in those studies were negative for campylobacter although there was an enrichment step, as in our study. We were able to detect campylobacter in three of the four slaughterhouses visited, after cleaning. No organic matter was visually detectable on surface samples (evisceration machines, conveyor belts), but feathers were observed on the rubber fingers of the defeathering machines in some cases. However, this had no effect on isolation percentages, and no difference was observed between the isolation percentages in samples from different sample sites. All samples of scald water taken before the start of processing on the second sampling day were negative for campylobacter. These results are in agreement with those of other authors (Borck and Pedersen, 2005). However, even after refilling the cleaned and disinfected scald tank, organic matter, such as feathers and dust, was still present in the water samples. The amount of water sampled may have been too small, and the limit of our detection method lower than the number of campylobacter cells present in the water in the cleaned scald tank. Another hypothesis is that campylobacter may not be detectable with our traditional microbiological method. Campylobacter is able to enter a viable but non-cultivable form (VNC) under stress conditions such as starvation and the hypo-osmotic stress encountered in aqueous environments (Rollins and Colwell, 1986). Further experiments should be developed to detect the viable but non-cultivable form of the pathogen and see if campylobacter was present in the scald tank water at the beginning of processing.

Although the mechanisms of resistance to disinfectant are poorly known, it is important to understand why certain bacteria survive after an apparently effective cleaning programme so that the procedure can be improved to avoid contamination of raw materials and products with pathogens and spoilage organisms present on surfaces in contact with food (Langsrud et al., 2003). Characterisation of the campylobacter strains isolated after cleaning would be promising in terms of control. Twenty-five isolates of *C. jejuni* but only one *C. coli* were collected after cleaning of equipment surfaces. There

362 was no statistically significant difference between the isolation percentages of *C. jejuni* and *C. coli*
363 before and after cleaning but this result may be biased due to the small number of isolates recovered
364 after cleaning: *C. jejuni* was detected in 9 (90%), and *C. coli* in only 1 (10 %) of the 10 positive
365 samples. Nevertheless, this suggests that *C. coli* may be more sensitive than *C. jejuni* or may be more
366 stressed and then, more difficult to recover after a cleaning procedure. Similarly, in another study
367 (Slader et al., 2002), it was suspected that the *C. coli* strain isolated from poultry before processing
368 was less robust than the strains of *C. jejuni* and could not survive processing. In our study, the
369 isolation method and more particularly the enrichment step, cannot be involved because strictly the
370 same procedure was used for samples collected before and after cleaning.
371 The aim of our study was to characterise and compare campylobacter isolates obtained before with
372 those obtained after cleaning. We therefore attempted to see if isolates collected after cleaning
373 showed reduced sensitivity to quaternary ammonium compounds. The choice of disinfectant
374 molecules in our study was limited to quaternary ammonium compounds: firstly, they are widely used
375 in the food-industry (and in at least two of the visited slaughterhouses), and secondly, they can be
376 used in the dilution agar method of MIC measurement. The distribution of quaternary ammonium MICs
377 was found to be monomodal and limited to a few values. Isolates collected after cleaning did not show
378 higher MICs to quaternary ammonium compounds than isolates collected before. However, firm
379 conclusions cannot be drawn due to the small number of isolates collected after cleaning. Also, MIC
380 measurement may not be the method of choice for studying disinfectant susceptibility, since the aim of
381 disinfection is primarily not to prevent growth, but to kill microorganisms. The MIC determination
382 method may not be suitable to reveal the distinctive features that enable *C. jejuni* strains to persist on
383 surfaces after cleaning. Nevertheless, in the food processing industry, disinfectants may be left on
384 surfaces with the resulting possibility of prolonged exposure of the micro-organism to the disinfectant
385 used (Bore and Langsrud, 2005), and for that reason, bacteria may be exposed to disinfectant
386 concentrations close to those used in MIC measurement.
387 The antibiotic phenotype of isolates obtained before and after cleaning procedures was determined,
388 because it has been widely suspected that disinfectant may select for antibiotic resistance (Russell,
389 2000). The cleaning of transport crates had no effect on the antibiotic resistance percentage or the
390 antibiotic phenotypes of isolates. The *C. jejuni* isolates obtained after cleaning of surfaces showed a
391 decreased degree of ampicillin resistance and multiresistance percentage compared to those obtained

before cleaning. This “in situ” observation does not suggest a cross-resistance or co-resistance between antibiotics and disinfectants. These results are in agreement with other experiments that did not show any cross- or co-resistance between biocides and antibiotics (Lear et al., 2006; Ledder et al., 2006). Our correlation matrix confirmed the absence of cross-resistance between antibiotics and quaternary ammonium MICs. At the same time, the validity of the data was confirmed by correlations between molecules of the same chemical family. Our results contradict our initial hypothesis and suggest that cleaning procedures do not select for antibiotic resistance in *C. jejuni* and *C. coli* species and seem to reduce the levels of resistance and multiresistance.

To survive in the environment, bacteria must respond to several stresses such as low nutrient concentrations and non-ideal growth conditions (Russell, 2003) and certain genotypes are likely to be better adapted to survive such stresses. Restriction fragment length polymorphism (RFLP) is a recommended method for typing poultry *Campylobacter* strains during the slaughtering process because of its low levels of strain non-typeability, acceptable levels of discriminatory power, and cost-effectiveness (Newell et al., 2001). Moreover, *fla*A typing has proven to be stable during storage (Wassenaar and Newell, 2000). Our results are probably biaised due to the few isolates collected after cleaning but still, they do suggest that only a limited number of genotypes are recovered after cleaning. Two genotypes were recovered from 3 to 4 different locations in slaughterhouse B, which was the most contaminated, strongly suggesting that these genotypes possess the ability to survive a routine cleaning procedure. *C. jejuni* is well known for its genome plasticity which may increase its potential to adapt and survive in hostile environments (Murphy et al., 2006). By the end of the working day, the slaughterhouse environment is heavily contaminated with campylobacter as a result of cross contamination of surfaces from the different flocks slaughtered. Evidence of both the repeated isolation of similar strains and the isolation of multiple genetically and phenotypically distinct strains within individual slaughterhouses, before cleaning, has been reported (Steele et al., 1998). Only similar genotype isolates were isolated after cleaning in our study. Quantitative analysis of campylobacter contamination on surfaces should be done to provide information about logarithmic decrease after cleaning . It would also allow to find out if strains recovered after cleaning were the most numerous before or if specific mechanisms are involved in their survival. The scope of investigations now needs to be broadened to include biofilms in which bacteria are relatively resistant to changes in environmental conditions, antimicrobial agents and host immune responses. The

hypothesis that *C. jejuni* cells form a biofilm to survive adverse conditions between animal hosts (Joshua et al., 2006) is attractive although another study indicated that *C. jejuni* in biofilms was susceptible to all the sanitizers tested (Trachoo and Frank, 2002).

In conclusion, our results show that *C. jejuni* and *C. coli* can survive overnight on the surfaces of slaughterhouse equipment after cleaning procedures. These procedures did not select for antibiotic resistance in *C. jejuni* in our study. Our results also suggest that specific genotypes have the ability to survive routine cleaning procedures. The mechanisms of survival of *Campylobacter* in the environment remain elusive which is one reason why these bacteria continue to pose a serious threat to public health. Understanding these survival mechanisms should facilitate the implementation of better targeted strategies and reduce the public health burden associated with *Campylobacter* infection.

Acknowledgements

We thank the staff at slaughterhouses for making this study possible. We are very grateful to I. Kempf for critical reading of the manuscript and comments.

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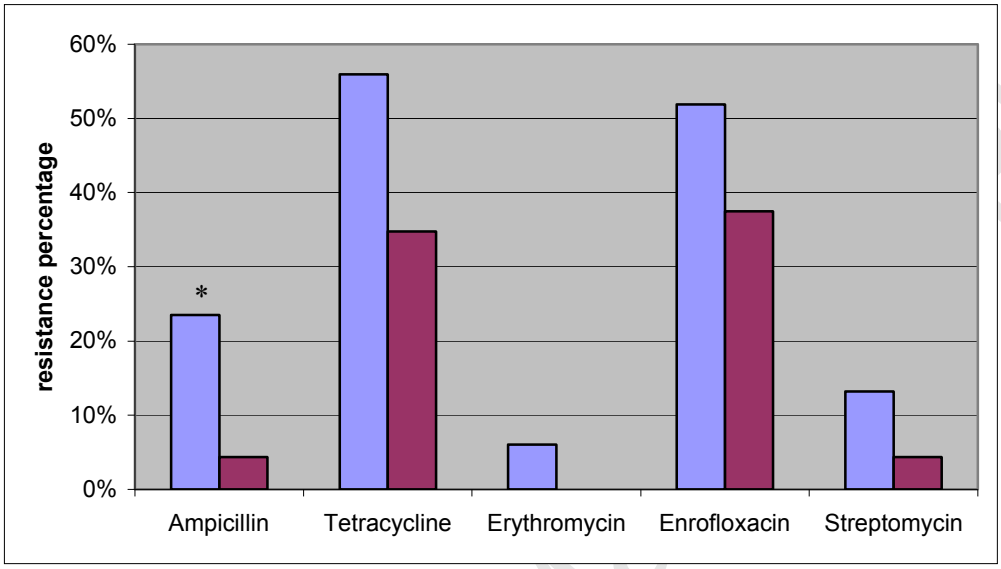
520

Fig. 1. Antibiotic resistance percentage of *C. jejuni* isolates from equipment swab samples before and after cleaning and disinfection



* significant difference ($p<0.05$)

Before cleaning & disinfection

After cleaning & disinfection

Fig. 2. Distribution of quaternary ammonium MICs of *C. jejuni* isolates collected before and after cleaning and disinfection on surfaces of slaughterhouse equipment

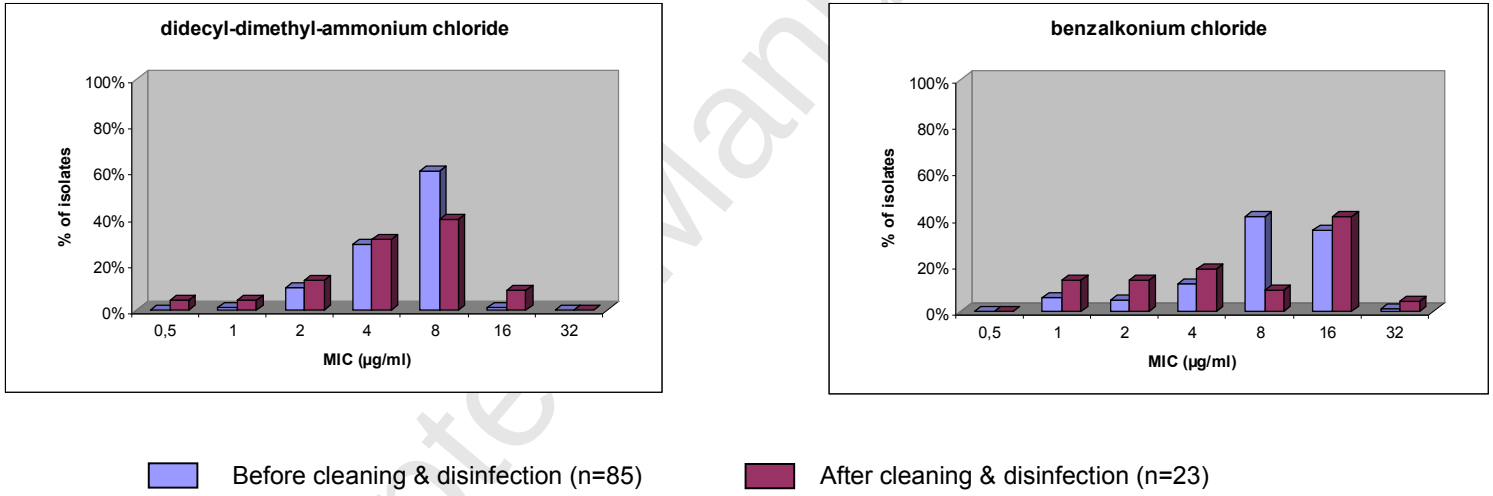
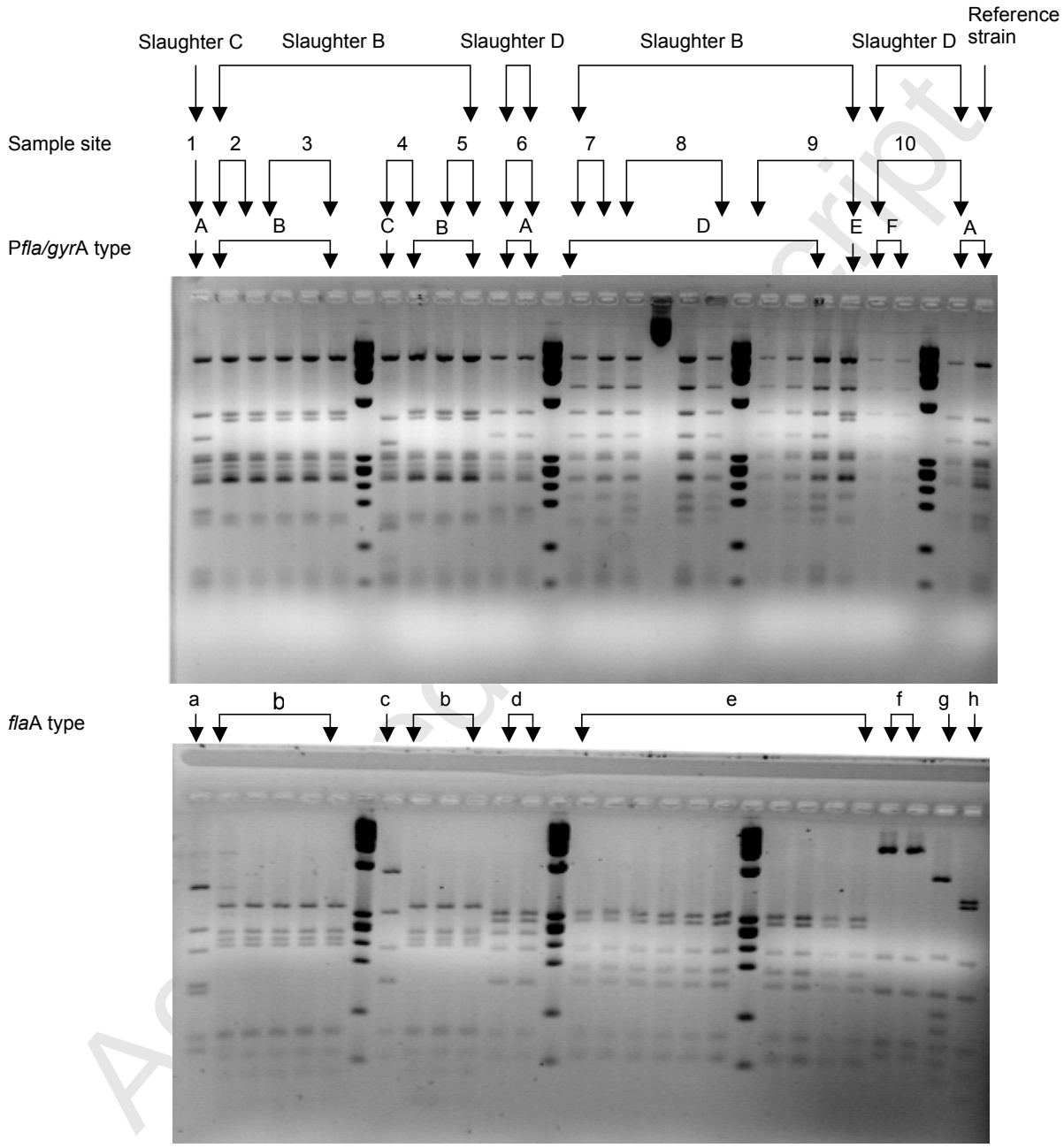


Fig. 3. P*fla/gyr*A and *fla*A profiles of *C. jejuni* isolates collected on surfaces after cleaning and disinfection



Sample sites 1- 2 : fingers of defeathering machines; sample sites 3-8 and 10: evisceration machines; sample site 9: conveyor belts. The Pfla/gyrA profile D not obtained for one isolate from slaughter B (sample site 8) was confirmed by further determination

Table 1.

List of primers used for identification and typing of campylobacter isolates

Target gene	Primers	Sequence	Annealing temperature (°C)	PCR-product (bp)	Reference
mapA	MDmapA1	5' CTA TTT TAT TTT TGA GTG CTT GTG 3'	52	589	Denis et al., 2001
	MDmapA2	5' GCT TTA TTT GCC ATT TGT TTT ATT A 3'			
*ceu*E	MDCOL3	5' AAT TGA AAA TTG CTC CAA CTA TG 3'	52	462	Denis et al., 2001
	MDCOL2	5' TGA TTT TAT TAT TTG TAG CAG CG 3'			
*pfl*A	PFLA1	5' GAG CTT GTT TTA AAC ACG GGT CGC 3'	60	2026	Ragimbeau et al.,1998
	PFLA2	5' TGA TAG TCA ATG GCC TTA GGT GCG 3'			
*gyr*A	GYR1	5' CTG GTT CTA GCC TTT TGG AAG C 3'	60	2661	Ragimbeau et al.,1998
	GYR2	5' GGA CAC TTA GCG ATG CTA ACC A 3'			
*fla*A	Pg50	5' ATG GGA TTT CGT ATT AAC 3'	45	1448	Alm et al., 1993
	RAA19	5' GCA CCY TTA AGW GTR GTT ACA CCT GC 3'			

Table 2.

Range of concentrations for antibiotics and disinfectants and breakpoints for antibiotics (according to CA-SFM 2007)

Antibiotic	Concentration (µg/ml)	Resistance breakpoint (µg/ml)
Ampicillin	2-32	> 16
Tetracycline	1-64	> 8
Erythromycin	0.25-32	> 4
Gentamicin	0.25-8	> 4
Enrofloxacin	0.25-16	> 1
Streptomycin	1-64	> 16
Benzalkonium chloride	0.5-32	
Didecyl-dimethyl-ammonium chloride	0.5-32	

Table 3.

Disinfectant products : composition, concentration and contact time used in the 3 visited slaughterhouses

		Product	Composition	Concentration	Contact time
Slaughter A	Transport crates	Deptil G4®	• Lauryl-dimethyl-benzyl ammonium chloride • Glutaraldehyde	1%	nd[1]
	Equipment	Galox-Azur®	• Lauryl-dimethyl-benzyl ammonium chloride • Glutaraldehyde	1%	20 minutes
Slaughter B	Transport crates	Baso agri+®	• Sodium hypochloride	nd	nd
	Equipment	Divosan 2000®	• Dimethyl-dialkyl ammonium chloride • Glutaraldehyde	3%	nd
Slaughter D	Transport crates	Hyprelva 4+®	• Didecyl-dimethyl ammonium chloride • Formaldehyde • Glutaraldehyde • Glyoxal	1.5%	nd
	Equipment	Indaluve®	• Poly(hexamethylene biguanide) chlorhydrate • Butylglycol	1%	15 minutes

[1]: not determined ie the slaughterhouse didn't provide the information

Table 4.

Results of sampling from equipment in the four slaughterhouses

	Cleaning and disinfection	No. of positive visits/ No. of visits	No of positive samples/ No. of samples	No of positive samples with *C. jejuni*/ No. of positive samples	No. of positive samples with *C. coli*/ No. of positive samples	No. of *C. jejuni* isolates	No. of *C. coli* isolates
Slaughter A	Before	1/1	6/6	5/6	2/6	20	4
	After	0/1	0/6	0	0	0	0
Slaughter B	Before	2/2	14/14	13/14	1/14	43	4
	After	2/3	7/22	7/7	0/7	19	0
Slaughter C	Before	1/1	3/6	3/3	0/3	5	0
	After	1/1	1/9	1/1	0/1	1	0
Slaughter D	Before	4/4	13/19	8/13	7/13	22	11
	After	2/4	2/19	1/2	1/2	5	1
Total	Before	8/8	36/45	29/36	10/36	90	19
	After	5/9	10/56	9/10	1/10	25	1

Table 5.

Antibiotic resistance profiles of *C. jejuni* isolates collected on slaughterhouse equipment surfaces before and after cleaning and disinfection

No. resistance	Resistance profile[1]	Before cleaning and disinfection			After cleaning and disinfection		
		No. of isolates	%	95% CI[2]	No. of isolates	%	95% CI
1	E	17	23	[14-35]	8	35	[16-57]
1	S	0	0	[0-5]	1	4	[0-22]
1	T	18	25	[15-36]	7	31	[13-53]
2	ES	4	6	[2-13]	0	0	[0-15]
2	TA	1	1	[0-7]	0	0	[0-15]
2	TAS	1	1	[0-7]	0	0	[0-15]
2	TE	1	1	[0-7]	0	0	[0-15]
3	TEA	13	18	[10-29]	1	4	[0-22]
3	TES	2	3	[0-10]	0	0	[0-15]
0	Susceptible	16	22	[13-33]	6	26	[10-48]
	Total	73	100		23	100	

[1] E: enrofloxacin resistant; S: streptomycin resistant; T: tetracycline resistant; A: ampicillin resistant; Susceptible to all antibiotics tested

[2] CI : confident interval

Table 6.

Samples sites of P*fla/gyr*A and *fla*A profiles of *C. jejuni* isolates collected on surfaces after cleaning and disinfection

	Visit	Sample site	P*fla*/*gyr*A type	*fla*A type
Slaughter B	1	Fingers of defeathering machines	B	b
		Evisceration machines	B	b
			C	c
	2	Evisceration machines	D	e
		Conveyor belts	D	e
			E	e
Slaughter C		Fingers of defeathering machines	A	a
Slaughter D	1	Evisceration machines	A	d
	2	Evisceration machines	F	f
			A	g