Molecular epidemiology of *Salmonella* and *Campylobacter* contamination of poultry during transport and slaughter

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TABLE OF CONTENT

ABBREVIATION LIST

A	absent
AFLP	amplified fragment length polymorphism
bp	base pairs
BPW	buffered peptone water
CDC	Center for Disease Control and Prevention
CFU	colony-forming units
CI	confidence interval
DI	differentiation index
Diassalm	diagnostic semi-solid *Salmonella* agar
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ERIC	enterobacterial repetitive intergenic consensus
EU	European Union
*fla*A	flagellin gene A PCR/restriction fragment length polymorphism
GBS	Guillain-Barré syndrome
IU	international unit
LPS	lipopolysaccharide
M	molar
mCCDA	modified cefoperazone charcoal deoxycholate agar
MLST	multilocus sequence typing
NC	not countable
ND	not done, not determined
NRSS	National Reference Centre for *Salmonella* and *Shigella*
NT	not typeable
OD_{600}	optical density at 600 nm
P	present
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
RA	reactive arthritis

RAPD	random amplification of polymorphic DNA
RDNC	routine dilution no conformity
rep-PCR	repetitive extragenic palindromic PCR
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
RV	rappaport-vassiliadis bouillon
SI	similarity index
SIPH	Scientific Institute of Public Health
spp.	species (plural)
TBE	Tris-borate-EDTA
TSA	tryptone soya agar
U	unit
UK	United Kingdom
UPGMA	unweighted-pair group method using arithmetic averages
US	United States
USA	United States of America
UV	ultra violet
VBNC	viable but non-culturable
vol/vol	volume/volume
WHO	World Health Organisation
wt/vol	weight/volume
XLD	xylose lysine deoxycholate

LITERATURE REVIEW

1. CAMPYLOBACTER

1.1. Introduction

Although campylobacters were not recognized as enteric pathogens in humans until the late 1970s, they have probably caused illness in men for centuries. These spiral bacteria have been known in veterinary medicine since the beginning of the 20th century. They were associated with abortion, sterility and dysentery in sheep, cattle or swine (Butzler, 2004). However, it took until 1968 to isolate *Campylobacter* from the stool of a patient with severe diarrhea and fever by a filtration technique (Dekeyser *et al.*, 1972). The later development of selective media, obviating the need to filter suspensions, made it possible for routine microbiological laboratories to isolate campylobacters from fecal samples. Reports from countries all over the world made it in the mid 1980s clear that *Campylobacter* is the most frequent cause of human bacterial enteritis (Butzler, 2004).

1.2. Taxonomy and characteristics

The genus *Campylobacter* was first proposed in 1963 by Sebald and Véron (1963) and included only two species, formerly classified as *Vibrio* spp. At present, the genus *Campylobacter* contains 17 species and 6 subspecies (Figure 1; On, 2001; Foster *et al.*, 2004). The genus *Campylobacter* is a member of the family of the Campylobacteraceae (Vandamme and De Ley, 1991) together with the genera *Arcobacter* and *Sulfurospirillum* (Vandamme *et al.*, 2005).

Campylobacters are Gram-negative spiral shaped rods, 0.2 to 0.8 µm wide and 0.5 to 5 µm long. Most species are motile with a characteristic corkscrew-like motion by a flagellum at one or both ends of the cell. Energy is obtained from amino acids or tricarboxylic acid cycle intermediates, not from carbohydrates. Carbohydrates are neither fermented nor oxidized. In general, campylobacters grow under micro-aerobic conditions with low levels of oxygen (ca. 5%) for energy production. Campylobacters are sensitive to several environmental conditions and are generally less resistant to environmental stress than other foodborne pathogens. Campylobacters are sensitive to drying and do not survive well on dry surfaces (Fernandez *et al.*, 1985). They are also sensitive to osmotic stress and will not grow at concentrations of 2%

NaCl (Doyle and Roman, 1982), whereas *Salmonella* Typhimurium for example, is able to grow at 4.5% NaCl (ICMSF, 1996). They are sensitive to acidic conditions and are not able to grow below pH 4.9 (Blaser *et al.*, 1980). Under less favorable conditions, campylobacters may form coccoid cells which are associated with a loss of culturability using traditional culture methods (Rollins and Colwell, 1986). It is still debated if this coccoid form is a non-viable, degenerative form or a dormant state that is non-culturable, but metabolically active and recoverable in suitable animal hosts (VBNC, Viable But Non-Culturable) (Moore, 2001).

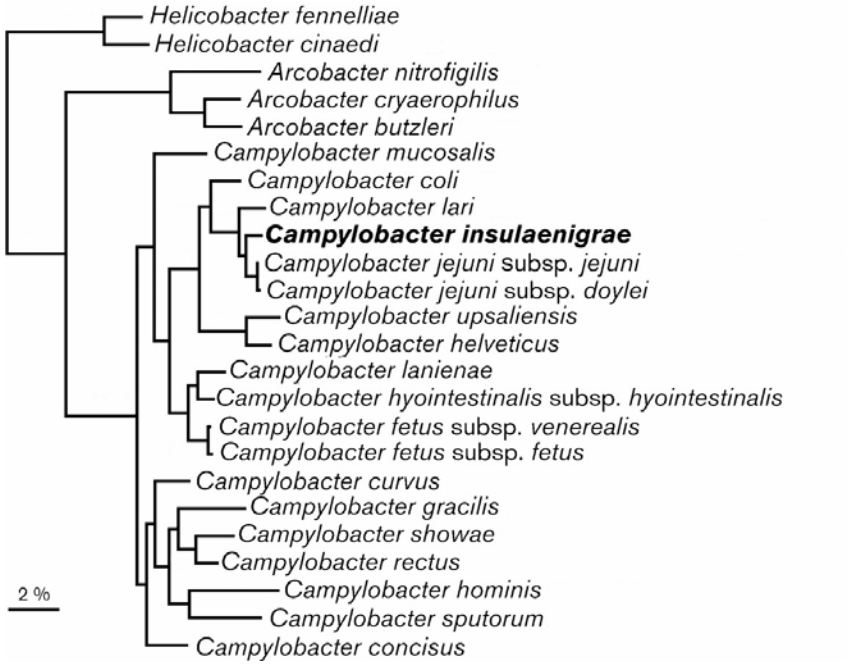

Figure 1. Unrooted tree, based on the 16S rRNA gene sequences, showing the phylogenetic relationships of *Campylobacter* spp., including the newest species *Campylobacter insulaenigrae*. Bar, 2% sequence divergence (adapted from Foster *et al.*, 2004).

Most campylobacters are not able to grow below 30°C and above 45°C. *Campylobacter jejuni*, *C. coli* and *C. lari* are the so-called thermophilic campylobacters because they are able to grow at a temperature of 37°C as the other *Campylobacter* species, but also at a temperature of 42°C. *C. upsaliensis* is also often described as thermophilic, but as not all strains are able to grow at a temperature of 42°C, it might be more appropriate to refer to this species as thermotolerant (Vandamme *et al.*, 2000). The pH for *Campylobacter* growth ranges from 4.9 to 9.0 with an optimal pH between 6.5 and 7.5 (ICMSF, 1996).

1.3. Clinical aspects

Campylobacteriosis is an acute diarrheal disease with clinical manifestations such as diarrhea, fever and abdominal pain. A definitive diagnosis can only be made by detecting campylobacters in the feces, since these symptoms are not distinctive from symptoms displayed by other organisms. In Belgium, *C. jejuni* subsp. *jejuni* (hereafter called *C. jejuni*) and *C. coli* account for 80% and 12% of human *Campylobacter* infections, respectively (Vandenberg *et al.*, 2004). There does not seem to be any clear difference in the clinical manifestations between infections caused by *C. jejuni* or *C. coli*. In one study *C. coli* was found to cause milder symptoms whereas in another the opposite was reported (Figura and Guglielmetti, 1988; Popovic-Uroic *et al.*, 1988).

In general, the infective dose is low. Infection has been induced with doses of 500-800 bacteria (Robinson, 1981; Black *et al.*, 1988). There are influencing factors such as virulence of the strain and the susceptibility of the patient. The mean incubation period of *Campylobacter* enteritis is 3 days with a range of 18 hours to 8 days (Skirrow and Blaser, 2000). The onset is often abrupt, with abdominal cramps followed by diarrhea. Other symptoms not always present are fever, headache, myalgia, dizziness, vomiting and rigors (Peterson, 1994a). The abdominal pain is continuous and intense and may mimic acute appendicitis. This pseudo-appendicitis is the most frequent reason for admission of *Campylobacter* enteritis patients to the hospital. The diarrhea is commonly profuse, watery and bile stained. After 1-2 days of diarrhea, fresh blood appears in the feces in about 15% of the patients (30% of hospital patients). The diarrhea lasts for 2-3 days, but the abdominal pain may persist for several more days (Skirrow and Blaser, 2000). Though the average duration of the illness is 4.6 days, according to a study covering nine outbreaks affecting about 1500 persons, one third of the patients were ill for more than 7 days (Millson *et al.*, 1991). In a Norwegian study, there was a mean duration of 3.8 days loss of school or work and 14.6 days for the presence of symptoms (Kapperud *et al.*, 1992b). Patients continue to excrete campylobacters in their feces for several weeks to months after recovery, unless the infection has been treated with antibiotics. Since the illness is usually self-limiting, antibiotic treatment is only necessary in immunocompromised persons, elderly or children. Erythromycin is the drug of choice due to its efficacy, low toxicity and low cost. Mortality is low and is usually confined to elderly patients or patients suffering already from another underlying disease.

According to Tauxe (1992) and Havelaar *et al.* (2000), the case-fatality ratio of campylobacteriosis is 3/10 000 and 1/10 000, respectively.

In some rare cases, campylobacteriosis is followed by severe complications such as the Guillain-Barré syndrome (GBS) and reactive arthritis (RA). GBS is the most frequent cause of acute flaccid paralysis in humans that usually develops following a gastrointestinal infection. One to three weeks after recovery, affected persons develop weakness of the limbs, the respiratory muscles and areflexia. At present, *C. jejuni* is the most widely recognized triggering agent of GBS. GBS is an autoimmune disease with the immune system mistakenly attacking myelin or axons of the peripheral nervous system. This immune attack may happen because the surface of *C. jejuni* contains polysaccharides that resemble gangliosides of the human nerve tissues. This resemblance has been termed ganglioside mimicry. The disease is self-limiting with a partial or complete recovery over weeks to months (Adams and Victor, 1993). The incidence of GBS ranges from 0.4 to 4 per 100 000 persons per year (Black *et al.*, 1988; Hughes and Rees, 1997; Mead *et al.*, 1999).

Reactive arthritis (RA) is an immune-mediated inflammation of the joints sometimes following gastrointestinal or urogenital infections. The symptoms are pain, stiffness, redness or swelling in the joints of the lower limbs (knees, ankles, toes) and more rarely in the joints of the upper limbs (wrists, fingers). The risk of developing reactive arthritis after campylobacteriosis is 1-3% (Havelaar *et al.*, 2000). The mean interval between the onset of campylobacteriosis and the appearance of pain and swelling of the joints is 14 days (Peterson, 1994b). The duration of reactive arthritis ranges from several weeks to several months, occasionally a year, but full recovery is the rule (Skirrow and Blaser, 2000).

1.4. Epidemiology

According to several studies, *Campylobacter* is the most reported bacterial cause of human gastroenteritis in the industrialized world (Tauxe, 1992). Until 2005, it was in Belgium the second most important cause after *Salmonella* (Figure 2; SIPH, 2006). In 2005, 66 human cases of campylobacteriosis per 100 000 habitants were reported (SIPH, 2006).

EFSA (2006b) reported a mean of 48 cases per 100 000 habitants in the 25 EU member states and Norway with incidences ranging from 0 cases per 100 000 habitants in Cyprus and Latvia

to 250 cases per 100 000 habitants in the Czech Republic. However, it is difficult to compare *Campylobacter* incidence rates between different countries due to differences in the monitoring and reporting procedures. In countries that have free health care and good laboratory support, such as in Northern Europe, the reported incidence ranges from 50 to 90 cases per 100 000 habitants per year (Friedman *et al.*, 2000; Norwegian Zoonosis Centre, 2006; EFSA, 2006b).

In most countries, including Belgium, a steady increase in the number of reported cases is observed (Figure 2; Figure 3). The increase in the 1980s can be explained by various factors such as increased physician awareness, increased culturing by laboratories, improved detecting methods and laboratory methods, whereas the increase observed since the 1990s more probably reflects a true increase in infections (Friedman, 2000). Some studies suggest that the true incidence is probably much higher than the reported cases. It is calculated that the true annual incidence is nearer 700-800 cases per 100 000 habitants per year in the United States and in the United Kingdom (Friedman *et al.*, 2000; Frost; 2001). The World Health Organization (WHO) estimates that ca. 1% of the European population is infected with *Campylobacter* each year (Notermans, 1994).

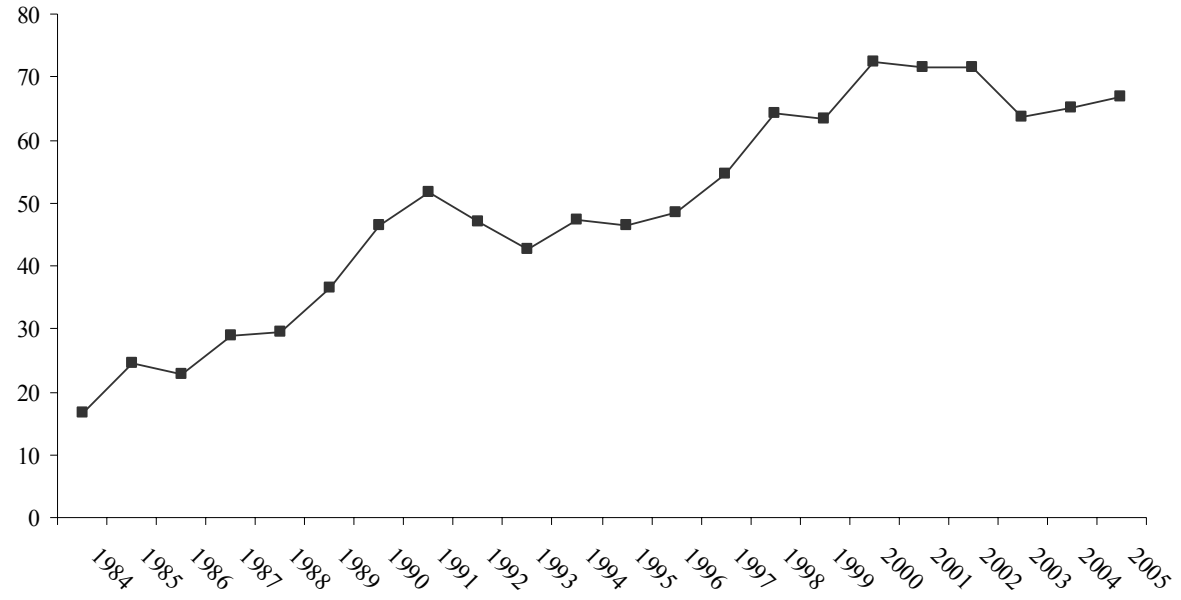

Figure 2. *Campylobacter* cases in Belgium from 1984 to 2005 per 100 000 habitants (adapted from SIPH, 2006).

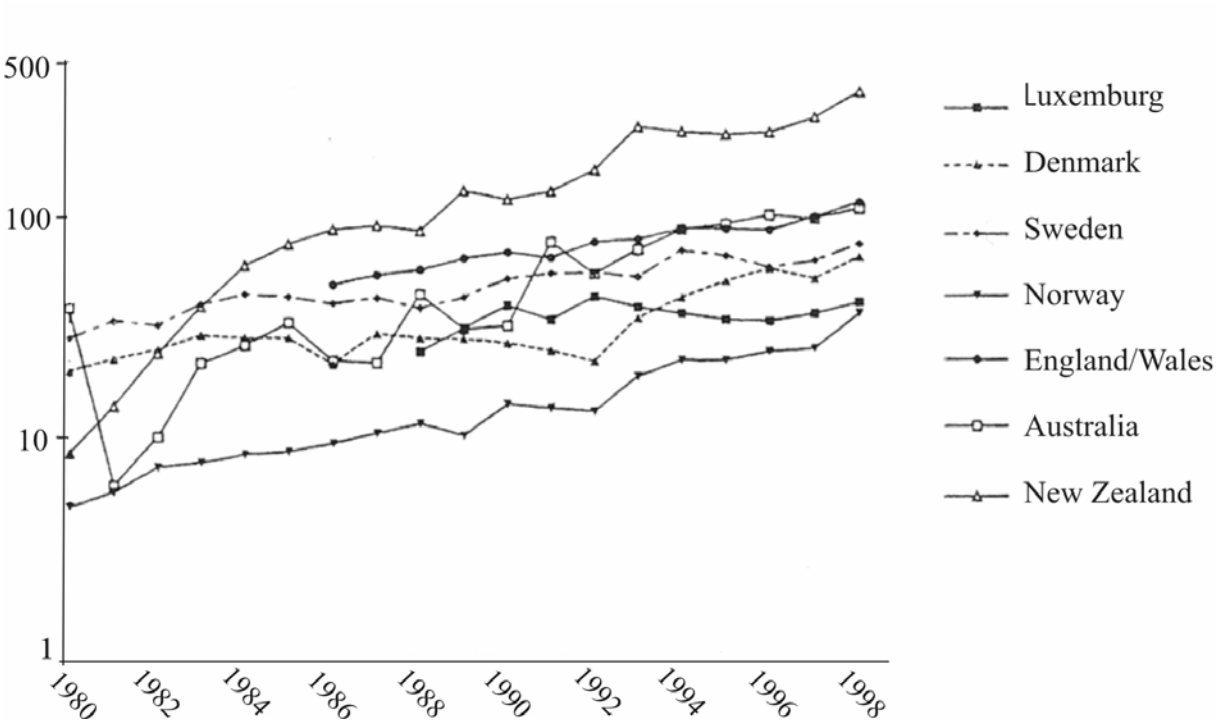

Figure 3. The evolution in *Campylobacter* cases in other countries from 1980 to 1998 per 100 000 habitants (Friedman *et al.*, 2000).

Some trends are observed in the *Campylobacter* incidence in the industrialized countries, including Belgium. First, *Campylobacter* affects all age groups but has a bimodal age distribution with a peak for children younger than 4 years and a second peak for young adults between 15 and 44 years (Friedman *et al.*, 2000). Hypotheses to explain this include oversampling in small children and increased foreign travel in young adults (Stafford *et al.*, 1996). Second, the *Campylobacter* incidence is 1.2 to 1.5 times higher in males than in females, which may be partly explained by a sex-specific behavior (Friedman *et al.*, 2002). A last trend is a seasonal distribution with a well-defined summer peak, observed in the United States as well as in European countries (Friedman *et al.*, 2000; Nylen *et al.*, 2002). This peak may vary from country to country and from year to year (Nylen *et al.*, 2002). Several hypotheses exist to explain the higher incidence of *Campylobacter* infection during the summer months. A first hypothesis is the variation in human behavior during the summer months such as increased animal contact, eating barbecue meals, and drinking or accidental ingestion of untreated water. A second explanation is a seasonal variation in the occurrence of *Campylobacter* in known reservoirs such as poultry flocks (Jacobs-Reitsma *et al.*, 1994; Refrégier-Petton *et al.*, 2001). However, since a few studies have demonstrated that the peak in human cases may occur simultaneously or even may precede the peak in flock colonization,

it can not be excluded that the peak in humans and broiler flocks are caused by a mutual still unidentified source (Kapperud *et al.*, 1993; Nylen *et al.*, 2002). A last hypothesis is that flies, which are more common during the summer months, are a potential source of infection (Ekdahl *et al.*, 2005; Nichols, 2005).

1.5. Sources of infection

In the 1970s and 1980s, unpasteurized milk and nonchlorinated water accounted for the majority of -often large- outbreaks. Nowadays, milk is pasteurized, sterilized or has undergone an ultra high heat treatment and water is disinfected before distribution in community systems (Friedman *et al.*, 2000). More recently, the majority of *Campylobacter* infections are not related to outbreaks but occur as sporadic infections. *Campylobacter* do not multiply on food, which explains the rarity of large outbreaks related to food. Since it is difficult to determine the source of an individual case, several case-control studies have been performed to identify the most likely sources of *Campylobacter* infections (Kapperud *et al.*, 1992a; Eberhart-Philips *et al.*, 1997; Neal and Slack, 1997; Rodrigues *et al.*, 2000; Studahl and Andersson, 2000; Sopwith *et al.*, 2003). Though different in the techniques applied and the array of hypotheses tested, they all indicate the same sources: handling or consumption of poultry meat, barbecue, drinking contaminated water, drinking bird-pecked milk, contact with pets and other animals and overseas travel (travelers' diarrhea). Case control studies have identified handling and consumption of poultry meat as a major risk factor for a variable percentage of cases ranging from 10% of the cases in Denmark to 50% in New Zealand and more than 70% of the cases at a US university (Eberhart-Philips *et al.*, 1997; Friedman *et al.*, 2000). Studies other than case-control studies have also shown the association between *Campylobacter* infection and poultry meat. In Belgium, the dioxin crisis in June 1999 resulted in the withdrawal of poultry meat from the Belgian market. In the study of Vellinga and Van Loock (2002), it was calculated that this withdrawal caused a decline of 40% in the number of Belgian *Campylobacter* infections. An outbreak of *Campylobacter* occurred in 1982 in Colorado among 11 of 15 people attending a party. The illness was associated with eating undercooked barbecued chicken (Franco, 1988). In another outbreak in Wales affecting 12 of 29 customers of a restaurant specialized in stir-fried food, illness was caused by eating stir-fried but undercooked chicken pieces (Evans *et al.*, 1998). Pearson *et al.* (2000) reported an outbreak of *C. jejuni* affecting 19 persons, who ate or worked in a college kitchen. The

outbreak was associated with poultry meat and an epidemiological investigation tracked the outbreak source to the farm of origin.

Studies have been performed to determine the presence of *Campylobacter* on retail chicken. It is difficult to compare the *Campylobacter* prevalence between different studies, since several sampling and isolation methods were applied. In Great Britain, the prevalence ranged from 68% to 83% (Kramer *et al.*, 2000; Harrison *et al.*, 2001; Jørgensen *et al.*, 2002; Meldrum *et al.*, 2004; Meldrum *et al.*, 2005; Meldrum *et al.*, 2006), which seems higher than the prevalence of 38% to 57% reported in Northern Ireland and Ireland (Madden *et al.*, 1998; Wilson, 2002, Whyte *et al.*, 2004). In the United States and Canada, *Campylobacter* was isolated from 62% to 82% of poultry carcasses (Zhao *et al.*, 2001; Bohaychuk *et al.*, 2006). In a German study, 70% to 77% of raw chicken legs were *Campylobacter* positive with a medium count of log 4.2 CFU/leg surface (Scherer *et al.*, 2006). Species identification revealed that *C. jejuni* was the most prevalent species isolated from raw poultry meat, with levels ranging from 77% to 98% (Kramer *et al.*, 2000; Zhao *et al.*, 2001; Dickins *et al.*, 2002; Jørgensen *et al.*, 2002; Whyte *et al.*, 2004).

Cross-contamination in the kitchen is also considered a risk for campylobacteriosis. A qualitative cross-contamination study showed that campylobacters are easily transferred from raw chicken products to cutting boards, plates and especially to hands (De Boer *et al.*, 1990). Cogan *et al.* (2002) quantified cross-contamination in a study where participants cut *Campylobacter* contaminated chicken carcasses in pieces. The results were that 85% of the hands and 80% of the cutting boards were contaminated, with 20% of the hands and 45% of the cutting boards at levels of more than 1000 CFU. Further cross-contamination from kitchen utensils to food was demonstrated by Kusumaningrum *et al.* (2004) with a mean transfer rate of 43% from stainless steel to cucumber slices. The packaging can also be considered as a risk factor, since Harrison *et al.* (2001) demonstrated that 3% of the outer packaging from raw poultry products was *Campylobacter* contaminated.

2. SALMONELLA

2.1. Introduction

In 1885, *Salmonella* was isolated from swine in association with the "swine plague" (Salmon and Smith, 1886). In 1888, an outbreak of gastroenteritis involving 58 persons was associated with the consumption of red meat. *Salmonella* Enteritidis, then named *Bacillus enteritis* was isolated from both the muscle tissue and the spleen of one of the patients who had died (Kelterborn, 1967). In the 1920s and 1930s, White (1926) and Kauffmann (1930, 1934, 1966) introduced the method for antigenic identification of the *Salmonella* group. According to this Kauffmann-White scheme, each *Salmonella* serotype is recognized by its possession of a particular lipopolysaccharide (LPS) or O antigen and a flagellar or H antigen. This led to the description of more than 2500 serotypes at present (Brenner *et al.*, 2000; Popoff and Le Minor, 1997; Popoff *et al.*, 2000, 2001, 2003, 2004). The extensive study of the organism has led to the recognition that *Salmonella* is one of the most common causes of human gastroenteritis.

2.2. Taxonomy and characteristics

The genus *Salmonella* belongs to the large family of Enterobacteriaceae. The taxonomy and the nomenclature have been the subject of debate in the past decennia. Nowadays, it is generally accepted that the genus *Salmonella* consists of three species, namely *Salmonella enterica*, *Salmonella bongori* and the recently discovered species *Salmonella subterranea* (Shelobolina *et al.*, 2004; Heyndrickx *et al.*, 2005; Tindall *et al.*, 2005). *Salmonella enterica* is subdivided into six subspecies (Figure 4). As mentioned in the preceding section, more than 2500 serotypes are currently described. Historically, serotypes were considered as species and therefore the serotype names were italicized. Nowadays, the former known *Salmonella enteritidis* is written as *Salmonella enterica* subsp. *enterica* serotype Enteritidis or simply *Salmonella* Enteritidis. Serovars belonging to the subspecies *enterica* are mainly associated with mammalians and birds, whereas the other serovars are mainly isolated from non-mammalians vertebrates or from the environment (Brenner *et al.*, 2000).

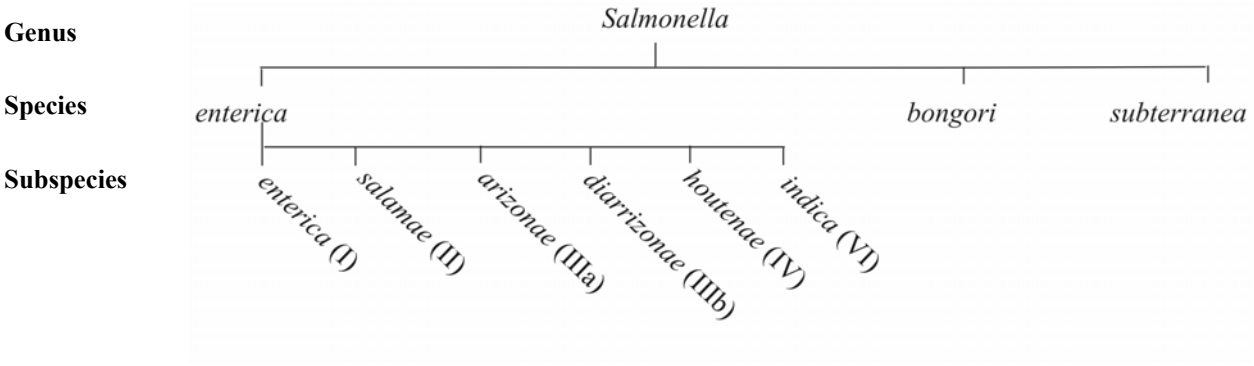

Figure 4. Currently accepted *Salmonella* nomenclature (Heyndrickx *et al.*, 2005; Tindall *et al.*, 2005).

Salmonella bacteria are Gram-negative rods that measure 0.7-1.5 by 2.0-5.0 μm. They are usually motile with peritrichous flagella and facultative anaerobic. Most species reduce nitrates to nitrites and they ferment glucose mostly with the formation of gas. Following biochemical characteristics are used for identification: urea not hydrolyzed, lysine and ornithine decarboxylation, and hydrogen sulphide production from thiosulphate on triple-sugar iron agar (ICMSF, 1996).

The growth of *Salmonella* spp. is dependent on several factors including temperature, pH, water activity and levels of nutrients present. In general, *Salmonella* grows at temperatures between about 5 and 46°C, with an optimum growth at approximately 37°C. *Salmonella* spp. decline during freezing, though the organism can survive for long time on frozen foods. *Salmonella* bacteria are killed by heat treatment. The pH for *Salmonella* growth ranges from 3.8 to 9.5 with an optimal pH between 7 and 7.5 (ICMSF, 1996).

2.3. Clinical aspects

The *Salmonella* serovars can be divided into groups based on their association with particular host populations. *Salmonella* serotypes which are almost exclusively associated with one particular host species are called the host-restricted serotypes (e.g. human *Salmonella* Typhi and poultry *Salmonella* Pullorum). Serotypes which are prevalent in one particular host species but can cause disease in other host species are the host-adapted serotypes (e.g. *Salmonella* Dublin causes disease in cattle but can also infrequently cause disease in other mammalian hosts). The last group of serotypes are the unrestricted or broad-host-range serotypes, capable of inducing disease in a broad range of unrelated host species (e.g. *Salmonella* Typhimurium and *Salmonella* Enteritidis) (Uzzau *et al.*, 2000).

Salmonella Typhi is the cause of typhoid fever with symptoms, such as sustained fever, headache, malaise, abdominal pain, enlargement of the liver and spleen and systemic infections. It is transmitted by ingestion of food or water contaminated with feces from an infected person. Therefore, typhoid fever is a problem in parts of the world with poor sanitation practices.

Other *Salmonella* serotypes are non-typhoid and cause less severe symptoms in humans. Symptoms of gastroenteritis occur between a few hours and five days following ingestion of the pathogen. The symptoms are diarrhea, abdominal pain, headache, nausea, mild fever and sometimes vomiting. The diarrhea is non-bloody and varies from a few, thin stools to massive evacuations with accompanying dehydration. The fecal excreta of infected persons contain large numbers of *Salmonella* at the onset of illness but the number of excreted bacteria decreases over time. The median duration of shedding is approximately one month in adults and seven weeks in children younger than five years (Hohmann, 2001). The disease is usually self-limiting and recovery occurs after a few days to a week. In some rare cases, the infection is followed by more serious complications especially in immunocompromised people, pregnant women, elderly and children. Approximately 5% of individuals with gastrointestinal illness caused by nontyphoidal *Salmonella* will develop bacteremia which is often accompanied with focal infections such as meningitis, septic arthritis, osteomyelitis, pneumonia and arteritis. About 2% of *Salmonella* infections are followed by reactive arthritis (see 1.3). According to the Center for Disease Control and Prevention (CDC) in the US, 0.04% of the estimated number of non-typhoidal *Salmonella* cases has a lethal outcome (Mead *et al.*, 1999).

Antibiotic therapy is only advisable for those who are severely ill, children younger than one year and for patients with risk for developing extraintestinal spread of infection. Antibiotics used to cure *Salmonella* infections are fluoroquinolones, third-generation cephalosporins, trimethoprim-sulfamethoxazole and ampicillin. However, resistance to the last two is common (Hohmann *et al.*, 2001).

Early studies indicated that the ingestion of more than 10^5 organisms is required to cause illness (McCullough and Eisele, 1951). In some instances, however, particularly when the vehicle has been water or fatty, small numbers (e.g. <100/g) of bacteria have been found in the contaminated food. For example, 1000 *Salmonella* Typhimurium in egg based ice cream and 100 *Salmonella* Eastbourne in chocolates caused illness (Armstrong *et al.*, 1970; Craven

et al., 1975). The difference in infectious dose appears to be associated with survival of the bacteria during transit through the stomach: water ingested at non-meal times has a minimal retention time, while fatty foods protect bacteria from stomach acids (ICMSF, 1996).

2.4. Epidemiology

In Belgium, 48 cases per 100 000 habitants were reported in 2005 (Figure 5). This is a remarkable decrease compared to previous years. For the first time since the beginning of national surveillance the *Salmonella* incidence is lower than the *Campylobacter* incidence (Figure 5).

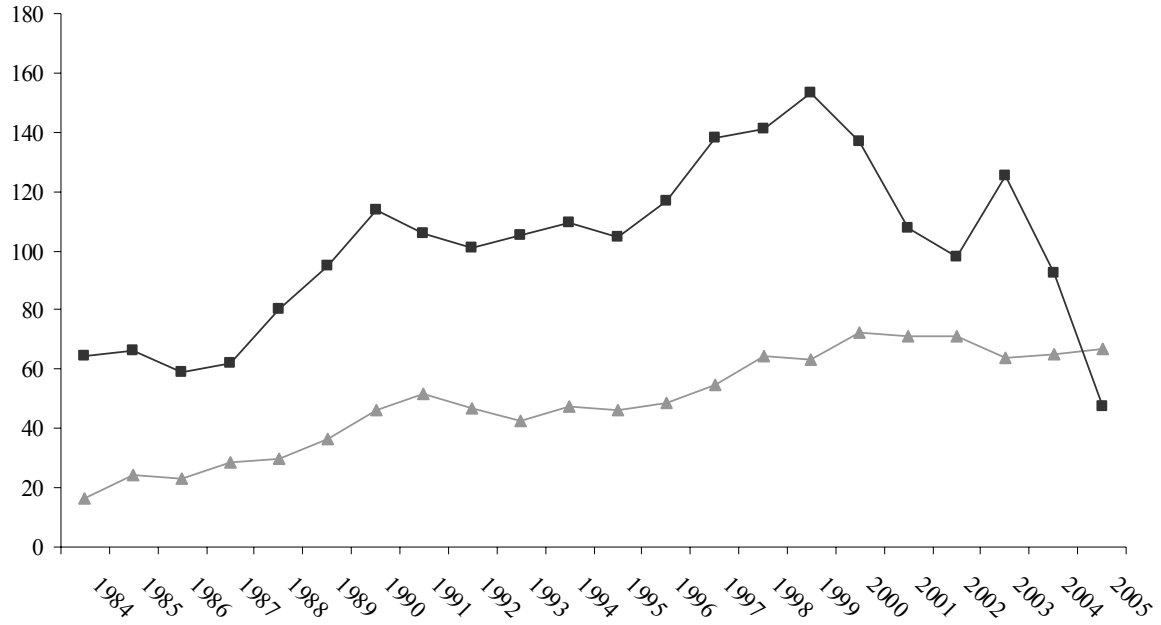

Figure 5. *Salmonella* cases (■) in Belgium from 1984 to 2005 per 100 000 habitants compared to the number of *Campylobacter* cases (▲) (adapted from SIPH, 2006).

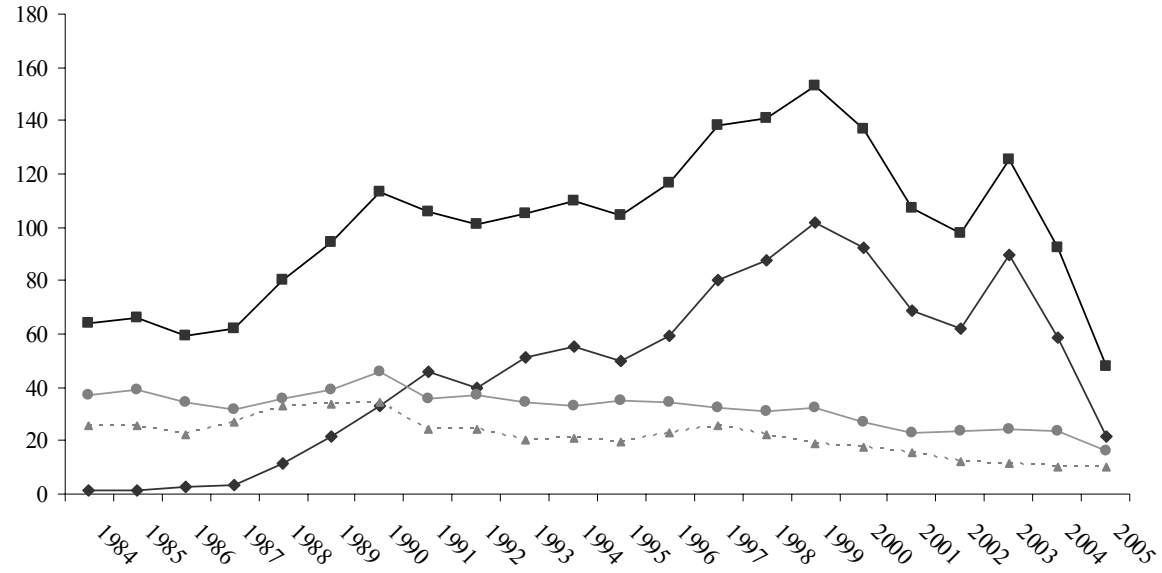

Figure 6. *Salmonella* cases (■) in Belgium from 1984 to 2005 per 100 000 habitants and the distribution of the most important serotypes : Enteritidis (♦), Typhimurium (●) and other serotypes (▲) (adapted from SIPH, 2006).

According to the EFSA report (2006b), the number of human *Salmonella* cases was 42 cases per 100 000 habitants in 2004 in the 25 EU member states, ranging from 7 cases per 100 000 habitants in Portugal to 300 per 100 000 habitants in the Czech Republic. However, these numbers should be interpreted with caution since the incidence in Belgium has been halved after 2004, so the incidences in the other countries are probably not up-to-date anymore, and the member states may use different monitoring and reporting procedures. On the other hand, as for *Campylobacter*, the number of cases is probably underreported. According to the Center for Disease Control and Prevention (CDC), the reported US incidence of 13 cases per 100 000 habitants must be multiplied by a factor of 37 to obtain a more realistic number of human *Salmonella* cases (Mead *et al.*, 1999).

As shown in Figure 6, the total number of Belgian human *Salmonella* cases shows an increase until 1999 and a remarkable decrease since 2000 (with the exception of 2003). This trend is also observed in other developed countries such as Sweden, Norway, Finland, Denmark, Ireland and Germany and is attributable to the serotype Enteritidis. Since the mid 1980s, public health laboratories in Europe and the US reported a dramatic increase in the number of human *Salmonella* Enteritidis (Rodrigue *et al.*, 1990). In 2004, *Salmonella* Enteritidis was the most common serotype in Europe with 76% of all *Salmonella* isolates belonging to this serotype (EFSA, 2006b). The second most important serotype, *Salmonella* Typhimurium, was

identified in 14% of all serotyped isolates which made it the second most important serotype. (EFSA, 2006b). The decrease observed recently could be related to the control programs in many countries (EFSA, 2006b). The peak in 2003, also observed in the Netherlands, can be explained by the avian flu in 2003 during which probably more *Salmonella* contaminated eggs were imported in Belgium and The Netherlands (Van Pelt *et al.*, 2004).

As observed for *Campylobacter*, children younger than 5 years old are the most affected age group. In Belgium, this age group represents 41% of all cases of salmonellosis reported (SIPH, 2006). A peak in the incidence in salmonellosis is seen in the late summer/autumn (SIPH, 2006; EFSA, 2006b).

2.5. Sources of infection

According to Mead *et al.* (1999), more than 95% of all *Salmonella* infections are foodborne. In the Netherlands, eggs and poultry meat are responsible for 39% and 21% of human salmonellosis cases, respectively, whereas human salmonellosis is caused by pork in 25% of the cases and by beef in about 10% of the cases (Van Pelt *et al.*, 1999). Eggs are undoubtedly the most important source of salmonellosis, especially in outbreaks where the serotype Enteritidis is involved (Cowden *et al.*, 1989; Henzler *et al.*, 1994; Mølbak and Neimann, 2002; Hald *et al.*, 2004). Poultry meat also contributes to the transmission of *Salmonella* to humans. It was recently shown in a study of the US that eating chicken outside of the home was the only significant risk factor for sporadic *Salmonella* Enteritidis infections (Kimura *et al.*, 2004). In Spain, there was recently a *Salmonella* outbreak with more than 2000 cases due to consumption of pre-cooked chicken of a particular brand (Lenglet, 2005).

In most countries, the prevalence of *Salmonella* contaminated retail chickens is much lower than *Campylobacter* contaminated poultry meat. In the United Kingdom, *Salmonella* prevalence of 5% to 30% on raw retail chicken are reported, which is comparable to the prevalence of 4% to 30% in Canada and the United States (Harrison *et al.*, 2001; Zhao *et al.*, 2001; Jørgensen *et al.*, 2002; Wilson, 2002; Meldrum *et al.*, 2004; Meldrum *et al.*, 2005; Bohaychuk *et al.*, 2006; Meldrum *et al.*, 2006). In a Belgian four-years study, *Salmonella* prevalence on poultry carcasses was 17% in 1993, 27% in 1994, 20% in 1995 and 27% in 1996 (Uyttendaele *et al.*, 1998). A decline in the *Salmonella* prevalence on poultry meat was noticed in several studies but only since the last few years. The *Salmonella* prevalence on

poultry carcasses in Northern Ireland rose from 7% in 1994 to over 20% in 1996, but more recently it declined to lower levels of 5-6% in 2000 (Wilson *et al.*, 1996; Wilson, 2002). Meldrum *et al.* (2004) found a significant decline between 2001 and 2004 with a rate falling from 8% to 5%. The prevalence of *Salmonella* on poultry carcasses in Southern Europe is higher than in the rest of Europe. In Spain and Portugal, the reported prevalence was 55% to 60% (Antunes *et al.*, 2003; Capita *et al.*, 2003).

Cross-contamination in the kitchen can also be considered as a risk for *Salmonella* infection, though the transfer rate from raw chicken products to hands and kitchen utensils and further to food is lower than for *Campylobacter* (De Boer *et al.*, 1990; Kusumaningrum *et al.*, 2004). Analogous as for *Campylobacter,* cross-contamination to the hands and cutting boards was quantified after volunteers had cut *Salmonella* contaminated chicken carcasses in pieces. The results were that 45% of the hands and the 35% of the cutting boards were contaminated with only 5% of the cutting boards at levels of more than 1000 CFU, which is less than for *Campylobacter* (see paragraph 1.5). However -in contrast to the results of *Campylobacter*- significant numbers of surfaces were still contaminated with low levels of *Salmonella* after cleaning and rinsing. Furthermore, growth can occur in *Salmonella* contaminated cloths stored overnight (Cogan *et al.*, 2002).

3. FLOCK COLONIZATION

3.1. Prevalence at farm level

Flocks can become colonized with *Campylobacter* from the age of two weeks (Jacobs-Reitsma *et al.*, 1995; Berndtson *et al.*, 1996b). Once introduced, campylobacters spread very quickly throughout the broiler house, probably via the drinking water system and by coprophagic behavior (Berndtson *et al.*, 1996a; Stern *et al.*, 2001a; Newell and Fearnley, 2003). Within a few days, all birds within the flock become colonized and shed campylobacters until slaughter age which is between five and six weeks (Jacobs-Reitsma *et al.*, 1995; Newell and Fearnley, 2003). It is often stated that campylobacters are harmless commensals in poultry and wild bird species. However, a severe disease, 'vibrionic hepatitis', was prevalent in the 1950s and 1960s in chickens in North America and Europe, which was apparently caused by *C. jejuni* (Corry and Atabay, 2001). In chickens, *Campylobacter* colonizes the mucus overlying the epithelial cells primarily in the ceca and the small intestine but may also be recovered from elsewhere in the gut and from the spleen and liver (Newell and Fearnley, 2003). Experimentally, the dose of campylobacters required to colonize chicks and chickens can be as low as 40 CFU, though it is dependent of the bacterial strain (Cawthraw *et al.,* 1996). Campylobacters can rapidly reach extremely high numbers in the cecal contents. Numbers in the region of 10^5-10^9 CFU/g intestinal contents have commonly been observed (Berndtson *et al.*, 1992; Berrang *et al.,* 2000; Corry and Atabay, 2001), although Wallace *et al.* (1997) reported levels higher than 10^{12} CFU/g in cecal contents.

The proportion of broiler flocks colonized with *Campylobacter* varies among countries, ranging from 3% in Finland (Perko-Mäkelä *et al.*, 2002) to more than 90% in the UK (Evans and Sayers, 2000) (Table 1). However, this variation may reflect, at least in part, different sampling and isolation methods applied. Nevertheless, there appears to be a lower prevalence of *Campylobacter* colonized flocks in the Nordic countries compared to the other European countries and the United States. The reason for this is still unknown, though climatic conditions, the distance between farms, and less intensive rearing practices may influence flock prevalence. Moreover, the poultry industry in the Nordic countries is more closely regulated than elsewhere in Europe (Newell and Fearnley, 2003). Several studies mention a seasonal variation in the prevalence of poultry flock colonization (Kapperud *et al.*, 1993;

Jacobs-Reitsma *et al.*, 1994; Wedderkopp *et al.*, 2001). This seasonal variation is expressed by a higher rate of colonization in summer than in winter. The reason for this seasonal variation is unknown but may reflect levels of environmental contamination. As mentioned in section 1.4, the peak in human campylobacteriosis incidence coincides with or even precedes the peak in flock colonization.

Table 1. Prevalence of *Campylobacter* colonized broiler flocks at slaughter age in various industrialized countries

Country	Prevalence (%)	Reference
Belgium	39[a]-67[b]	Herman *et al.*, 2003
The Netherlands	82	Jacobs-Reitsma *et al.*,1994
	45	van de Giessen *et al.*, 2006
United Kingdom	76	Humphrey *et al.*, 1993
	> 90	Evans and Sayers, 2000
Denmark	45	Hald *et al.*, 2000
	46	Wedderkopp *et al.*, 2000
	43	Wedderkop *et al.*, 2001
	50	Bang *et al.*, 2003
Finland	3	Perko-Mäkelä *et al.*, 2002
Norway	18	Kapperud *et al.*, 1993
Sweden	27	Berndtson *et al.*, 1996b
	<10	Newell and Fearnley, 2003
	17	Hansson *et al.*, 2004
Canada	60	Nadeau *et al.*, 2002
United States	88	Stern *et al.*, 2001b

[a] : prevalence determined by collecting cecal droppings in the poultry house at the age of 6 weeks just before slaughter
[b] : prevalence determined by collecting ceca (from the same flocks as in [a]) in the slaughterhouse at the age of 6 weeks

The pathogenicity of *Salmonella* depends on the serotype, the strain, the susceptibility of the birds and the age of the birds (Barrow, 2000). *Salmonella* Pullorum and *Salmonella* Gallinarum are respectively responsible for the Pullorum disease and fowl typhoid disease. Pullorum disease causes weakness, white diarrhea and a high mortality rate (50% to 100%) among embryos and young chicks. Fowl typhoid is a disease of mature fowl that results in

either acute enteritis with greenish diarrhea or a chronic disease of the genital tract that reduces egg production. Certain strains of other serotypes are also able to cause disease. After natural infection with *Salmonella* Enteritidis of broilers, indurated yolk sac remnants, pericarditis, necrotic foci and petechiae in the liver have been observed (O'Brien, 1988). As the birds age, they become more resistant to *Salmonella*, though *Salmonella* can colonize the intestines or cause a systemic infection in the absence of disease. Experimental infection of adult birds results in fecal shedding which is much lower than after infection of young chickens. It has been supposed that the birds are more resistant to *Salmonella* due to the presence of a more complex intestinal flora when the birds become older (Barrow, 2000). The intestines, especially the ceca are the primary sites of colonization for *Salmonella* (Fanelli *et al.*, 1970). *Salmonella* can also be isolated from a variety of organs including the spleen, liver, gall bladder, heart, ovaries and oviducts (Desmidt *et al.*, 1997).

In contrast to *Campylobacter*, *Salmonella*-positive birds can become (apparently) free of infection. For example, in a Belgian study in which 18 flocks were followed from hatching to slaughter, 10 flocks received a *Salmonella*-positive status. Nine of these flocks were already positive after two weeks of rearing, the remaining flock became positive after four weeks. The number of positive flocks dropped to six after six weeks of rearing (Heyndrickx *et al.*, 2002). In a study of Bolder *et al.* (1999), groups of 21 broilers were inoculated with 10^8 CFU *Campylobacter* or *Salmonella* and sampled for six weeks. During this period, all broilers shed campylobacters with a mean of approximately 10^7 CFU/g, the number of *Salmonella* shedding birds decreased from 21 in the beginning of the experiment to 14 birds at six weeks with mean fecal counts of 3×10^4 CFU/g in shedding broilers. However, when *Salmonella* is undetectable in the feces of the birds, it is possible that they are still carriers (Van Immerseel *et al.*, 2004). They can become shedders again when the immune response of the chicken is lowered due to stress or concurrent diseases. Rigby and Pettit (1980), for example, have shown that birds can change from *Salmonella* carriers to shedders during transport which is known to be stressful.

In Table 2, an overview of the prevalence of *Salmonella* colonized flocks in various industrialized countries is given. As explained above, birds can be carriers without shedding the organisms and flock prevalence decreases with age. Therefore the type of sample and the age of the birds are included in Table 2. The within flock-prevalence for *Salmonella* is

variable, ranging from 5 to 43% in Japanese broiler flocks (Limawongpranee *et al.*, 1998). No seasonal trends have been reported (Jacobs-Reitsma *et al.*, 1995; Wedderkopp *et al.*, 2001).

Only a few studies have investigated the possible correlation between colonization with *Campylobacter* and *Salmonella*. A positive correlation between *Campylobacter* and *Salmonella* colonization within Dutch flocks was found by Jacobs-Reitsma *et al.* (1995) and Jacobs-Reitsma (1995). In contrast, in the study of Wedderkopp *et al.* (2001) no significant correlation between *Campylobacter* and *Salmonella* colonization was found.

Table 2. Prevalence of *Salmonella* colonized broiler flocks in various industrialized countries

Country	Prevalence (%)	Type of sample	Age of birds	Reference
Belgium	50	overshoes	2 weeks	Heyndrickx *et al.*, 2002
	33	overshoes	6 weeks	Heyndrickx *et al.*, 2002
The Netherlands	27	cecal content	at slaughter	Jacobs-Reitsma *et al.*, 1995
	25	fecal samples	3 - 4 weeks	van de Giessen *et al.*, 2006
	12	fecal samples	6 weeks	van de Giessen *et al.*, 2006
France	70	environmental samples	4 - 6 weeks	Rose *et al.*, 1999
Spain	30	fecal samples	at slaughter	Carramiñana *et al.*, 1997
Denmark	17	cecal content	3 weeks	Angen *et al.*, 1996
	6	overshoes	3 weeks	Wedderkop *et al.*, 2001
	< 5	fecal samples	3 - 4 weeks	Wegener *et al.*, 2003
Sweden	0	cecal content fecal samples	4 - 5 weeks	Wierup *et al.*, 1995
Canada	77	litter and water samples	1 - 8 weeks	Renwick *et al.*, 1992
USA	5	fecal samples	6 - 7 weeks	Jones *et al.*, 1991b
Japan	64	cecal content	at slaughter	Limawongpranee *et al.*, 1999

3.2. Vertical transmission

The theory of vertical transmission for *Campylobacter* has been a controversial issue. *Campylobacter* is present in the reproductive organs and semen which could lead to vertical transmission of *Campylobacter* from the hen to the chick (Buhr *et al.*, 2002). Based on a correlation between hatchery and broiler flock colonization, Pearson *et al.* (1996) concluded that vertical transmission was an external source of *Campylobacter* broiler flock colonization. Cox *et al.* (1999) came to the same conclusions by genotyping the strains isolated from breeder flocks and their progeny. In contrast, other studies in which the strains from the broiler flocks and the parent flocks were compared suggest that there is little likeliness of vertical transmission (Chuma *et al.*, 1997a; Petersen *et al.*, 2001b). Moreover, if colonization takes place by vertical transmission, it would be expected that campylobacters would be detected in an affected flock immediately after hatching as observed with the vertical transmission of *Salmonella* in chickens. However, there is a delay of two to three weeks before the birds become colonized with *Campylobacter*. It is possible that small numbers of *Campylobacter* may be present in the hatching chick, but that the growth is constrained by maternal antibodies (Sahin *et al.*, 2001, 2003). More sensitive molecular detection techniques such as Southern blot hybridization and PCR have detected *Campylobacter* DNA in the cecal contents of 18-day-old embryos, newly hatched chicks and broilers less than three weeks old (Chuma *et al.*, 1997b). Since no *Campylobacter* was recovered from the cecal contents by conventional culture with selective enrichment, it has been suggested that the organisms were dead or in a nonculturable state. Nowadays, the general tendency is to control the horizontal route that appears to be the major risk for broiler flock colonization and then, to determine the role of vertical transmission if there are any problems left (Newell and Fearnley, 2003).

There are two possible routes of *Salmonella* contamination of intact hatching eggs: vertical transmission (transovarian route) or horizontal transmission (trans-shell route). Vertical transmission can originate from an infection of the reproductive organs of the breeder hen via systemic infection, or from an ascending infection from the cloaca to the vagina and lower regions of the oviduct. Via vertical transmission, the yolk, the yolk membrane or the albumen surrounding it, are directly contaminated as a result of *Salmonella* infection of the reproductive organs before the eggs are covered by the shell. Egg shell membrane and egg shell are produced in the lower reproductive tract. These compartments of the egg also may be

contaminated during egg development. Via the horizontal route, *Salmonella* penetrates through the egg shell after oviposition via for example fecal contamination on the shell. However, it is difficult to distinguish between contamination during formation of the egg and contamination after oviposition (De Buck *et al.*, 2004; Messens *et al.*, 2005). Finally, one-day-old chicks can also become colonized in the hatchery, for example, by contaminated equipment. However, as demonstrated by Heyndrickx *et al.* (2002) vertical transmission is nowadays of less importance mainly due to the vaccination of breeder flocks. Horizontal transmission is the main determinative factor for colonization of broiler flocks.

3.3. Horizontal transmission

The houses used for rearing broilers can largely be considered as closed environments. However, *Salmonella* and *Campylobacter* are present in the environment in and around broiler houses. Many studies have been undertaken to identify the risk factors for *Salmonella* or *Campylobacter* colonization. In the following paragraphs, each potential source is reviewed.

Feed and drinking water

It is widely accepted that feed is not a potential source of *Campylobacter* transmission to poultry. The dry conditions of feed are considered lethal to *Campylobacter* (Humphrey *et al.*, 1993; Berndtson *et al.*, 1996b). In contrast, several studies have linked contaminated feed to the occurrence of *Salmonella* in poultry (Primm, 1998). Analyses of commercially manufactured feeds confirmed that both feed ingredients and dust can be sources of *Salmonella* contamination in feed mills (Davies and Wray, 1997; Jones and Richardson, 2004). Heyndrickx *et al.* (2002) demonstrated that 3.5% of fresh feed samples tested positive for *Salmonella*. Moreover, *Salmonella* can survive for long periods of time in feed (e.g. 16 months at 25°C and 51% relative humidity) (William and Benson, 1987).

Although the drinking water in poultry houses of colonized poultry flocks is often contaminated with *Salmonella* or *Campylobacter*, this water contamination usually follows flock colonization rather than preceding it (Heyndrickx *et al.*, 2002; Newell and Fearnley, 2003). According to most studies, the water source is a low risk factor for flock colonization

with *Salmonella* or *Campylobacter* (Humphrey *et al*., 1993; Jacobs-Reitsma *et al.*, 1995; Rose *et al.*, 1999; Gradel and Rattenborg, 2003; Newell and Fearnley, 2003; Cardinale *et al.*, 2004). This is in contrast with a study of Pearson *et al.* (1993), in which was reported that a *Campylobacter* serotype persisted for at least 18 months on a poultry farm. The source was shown to be the farm's water system; campylobacters were found from the bottom of the 30-m borehole to the biofilm of the pipework within the poultry houses. According to a recent study, waterborne protozoa have strong potential to act as protective reservoirs for *C. jejuni* in the drinking water systems of poultry houses (Snelling *et al.*, 2005). Experimental cocultivation of *C. jejuni* with such protozoa appears to reduce the susceptibility of the bacteria to chlorine as well as to certain disinfectants (Snelling *et al.*, 2005). These factors may explain the observation by Stern *et al.* (2002) that chlorination of drinking water had no effect on the *C. jejuni* colonization of broilers.

Many studies have investigated the possibility of acidification of feed or water to reduce *Campylobacter* and *Salmonella* colonization of broilers. These studies, reviewed by Doyle and Erickson (2006) and Van Immerseel *et al.* (2006), yield conflicting results. It appears that the way of administration, the type of acid and the used concentration are very important. Furthermore, if the infection pressure is high or when the chickens are highly stressed, colonization is not always affected by this treatment.

Broiler house cleaning and disinfection

The carry-over from a *Campylobacter* or *Salmonella* colonized flock to a new flock in the same house seems an obvious source. *Salmonella* is frequently isolated in poultry houses after the cleaning and disinfection process (Higgins *et al.*, 1981; Davies and Wray, 1996; Rose *et al.*, 2000; Heyndrickx *et al*, 2002). A fundamental error is over-dilution or inconsistent application of disinfectants (Davies and Wray, 1996). Despite this, there are no published reports of *Campylobacter* isolation from emptied, cleaned and disinfected poultry houses. Consequently, infection is not predictable from the *Campylobacter* status of the previous flock in the house (Evans and Sayers, 2000). Negative flocks can follow positive flocks (Berndtson *et al.*, 1996b), positive flocks can occur in newly constructed houses (Gregory *et al.*, 1997), and sequential positive flocks can be colonized by different genotypes (Newell and Fearnley, 2003). These studies suggest that routine house cleaning and disinfection are largely adequate for *Campylobacter* decontamination. Even more, in the case that the litter is not

removed from the poultry houses and that the poultry houses are not cleaned and disinfected between flocks as in the United States, this does not cause an increase in *Campylobacter* colonized flocks (Newell and Fearnley, 2003). Payne *et al.* (1999) showed that chicks reared on litter removed from a *Campylobacter*-positive house did not become colonized over a 7-week period. This indicates the poor survival or colonization capacity of these organisms. In contrast, flock positivity is linked to a too short down period between rotating flocks (Hald *et al.*, 2000, Wedderkopp *et al.*, 2000). It is advisable to maintain a down period of at least two weeks (Hald *et al.*, 2000).

Human traffic and activities

The main human traffic in and out of a broiler house is the farm staff for the purpose of routine animal husbandry. *Salmonella* and *Campylobacter* can be carried into the house via boots, clothes and equipment (Davies *et al.*, 1997; Heyndrickx *et al*, 2002; Herman *et al.*, 2003). Therefore, introducing a hygiene barrier with an anteroom and walk-over benches, using boot dips or better house dedicated footwear, washing hands with antiseptic soap, changing clothes, cleaning the equipment that is brought into the house, and minimizing visits are all measures to improve hygiene and to reduce the possibility of flock colonization (Humphrey *et al.*, 1993; Kapperud *et al.*, 1993; Berndtson *et al.*, 1996b; van de Giessen *et al.*, 1996; Hald *et al.*, 2000; Heyndrickx, 2002; Gradel and Rattenborg, 2003, Newell and Fearnley, 2003; Cardinale *et al.*, 2004). The extent of *Campylobacter* contamination in the environment of the broiler house will obviously contribute to the risk of introducing *Campylobacter* into the broiler house. Studies of Hiett *et al.* (2002) and Bull *et al.* (2006) have demonstrated that *Campylobacter* isolates from puddles, recovered before flock colonization, were of the same genotype as isolates subsequently isolated from the broiler flock. Therefore, clean and intact concrete aprons around the broiler house can reduce the risk of flock colonization (Newell and Fearnley, 2003).

Thinning of the flock, which is reducing bird density within the broiler house, is a common procedure in many European countries, including Belgium. This practice enables higher productivity and provides the market of birds of different weights. Thinning or partial depopulation occurs normally at the age of 35 days, depending on the size and weight of the birds. During thinning, the doors of the poultry house are opened and the catching crew and the catching equipment enter the poultry house without any hygiene measures. Ramabu *et al.*

(2004) found that trucks, forklifts, pallets, crates and drivers' and catchers' boots were all *Campylobacter* contaminated. According to Hald *et al.* (2001), thinning is a major risk factor in the introduction of *Campylobacter* into the broiler house. All flocks which were *Campylobacter* negative at the time of partial depopulation became *Campylobacter* colonized by the following week when the rest of the flock was sent for slaughter. This conflicts with another study in which partial depopulation was no risk factor for introducing *Campylobacter* and the results of the study above were explained as age related (Russa *et al.*, 2005). The longer the birds are kept on the farm the higher the possibility the birds become colonized (Berndtson *et al.*, 1996b)

Rodents, insects and wild birds

Though conventionally reared poultry flocks are kept in closed poultry houses, some animals such as rodents and insects may have free access to the house. The significance of rodents, mice in particular, as vectors and reservoirs of *Campylobacter* and *Salmonella* has been shown by several studies (Henzler and Opitz, 1992, Davies and Wray, 1995; Davies and Wray, 1996; Rose *et al.*, 2000; Hiett *et al.*, 2002; Meerburg *et al.*, 2006). Henzler and Opitz (1992) have shown that less than 15 *Salmonella* bacteria are enough to infect a mouse and that mice droppings can contain up to 2.3 x 10^5 CFU/dropping. Mice produce *Salmonella* contaminated droppings for two to five months (Davies and Wray, 1995; Henzler and Opitz, 1992). No such data are available for *Campylobacter*. Since most farms apply rodent control programs, some studies consider rodents not longer as a significant risk factor for introducing *Salmonella* and *Campylobacter* in poultry houses (Berndtson *et al.*, 1996b, Kapperud *et al.*, 1993).

More difficult to control are insects. Several hundreds of flies per day pass trough the ventilation system into the broiler house according to Hald *et al.* (2004). Flies and beetles in and around poultry houses have been reported to carry *Campylobacter* and *Salmonella* (Rosef and Kapperud, 1983; Jacobs-Reitsma *et al.*, 1994; McAllister *et al.*, 1994; Gray *et al.*, 1999; Olsen *et al.*, 2000; Mian *et al.*, 2002; Bates *et al.*, 2004; Hald *et al.* 2004; Skov *et al.*, 2004; Strother *et al.*, 2005). Under experimental conditions, flies can become infected by *Campylobacter* colonized chickens and are able to transmit the bacteria to *Campylobacter*-free flocks (Shane *et al.*, 1985). Chickens consuming one *Campylobacter* or *Salmonella*

contaminated beetle became infected by *Campylobacter* or *Salmonella*, respectively (McAllister *et al.*, 1994; Strother *et al.*, 2005). However under non-experimental conditions, it is still not clear if insects cause *Campylobacter* colonized birds or *vice versa*. According to Shreeve *et al.* (2002), the carry-over from one flock to a subsequent flock in the same broiler house is relatively infrequent, which led to the conclusion that insects seem to be a relatively low risk (Newell and Pearson, 2003). This is confirmed by a recent study of Templeton *et al.* (2006), which demonstrated that *Campylobacter* does not survive for extended periods (less than 72 h) in or on darkling beetles and is consequently not a source of the carry-over of *Campylobacter* in flocks subsequently raised in the same poultry house.

Wild birds have, under good management practices, no access to the broiler houses. However, contaminated droppings can be brought into the house by footwear, clothing or material. Though many wild birds are colonized with *Campylobacter* or *Salmonella* (Waldenström, 2002; Pennycott *et al.*, 2006), the genotypes of the isolates from wild birds and from broilers are seldom the same (Petersen *et al.*, 2001a; Pennycott *et al.*, 2006). This suggests that the importance of wild birds as a reservoir of infection is limited.

Pet animals and livestock

A last risk that should be considered is the presence of pet animals and livestock such as pigs, cattle and sheep around the broiler house. These animals are unlikely to enter the house, but they may excrete *Campylobacter* or *Salmonella*. This can result in the contamination of boots, clothes or equipment taken into the house. Liebana *et al.* (2002) found that the cattle located in the proximity of the feed mill of a broiler house were colonized with the same *Salmonella* strain as the broilers. The same strain was also isolated from the feed mill. Identical *Campylobacter* strains were found in cattle next to the broiler house and subsequently in the broiler house (Gregory *et al.*, 1997, Newell and Fearnley, 2003). However, transmission of *Campylobacter* between pigs and poultry on mixed-species farms occurs infrequently according to Boes *et al.* (2005). Moreover, replacing the livestock by new broiler houses to avoid loss of income is not an option, since it has been demonstrated that an increasing number of poultry houses are associated with a higher risk of colonization with *Campylobacter* and/or *Salmonella* (Angen *et al.*, 1996, Skov *et al.*, 1999, Doyle and Erickson, 2006).

4. TRANSPORT AND SLAUGHTER OF BROILER FLOCKS

4.1. Transport

At the age of approximately six weeks, broilers are loaded in containers and transported to the slaughterhouse. Flocks are sometimes partially depopulated at the age of five weeks, which can be considered as a risk factor for introducing *Campylobacter* as described in 3.2.

Different studies have shown that the cleaning and disinfection process is often inadequate in eliminating *Campylobacter* and *Salmonella* from containers. In the study of Slader *et al.* (2002) and Hansson *et al.* (2005), 60% of the cleaned and disinfected transport containers sampled were *Campylobacter* contaminated. Slader *et al.* (2002) examined the effects of detergent and various disinfectants on the contamination level in the crates. Although the number of *Campylobacter* contaminated crates was reduced, none of the treatments eliminated campylobacters completely from the crates. Newell *et al.* (2001) found that on one occasion carcasses from a *Campylobacter*-free flock were contaminated with a *Campylobacter* strain that was isolated from the crates prior to loading of the birds. In the study of Rigby *et al.* (1982), 99% of the cleaned and disinfected containers examined were still contaminated with *Salmonella*. More recently, *Salmonella* was isolated from 13% to 87% of cleaned and disinfected containers at eight Danish poultry slaughterhouses (Olsen *et al.*, 2003). According to Rigby *et al.* (1980) and Corry *et al.* (2002), more crates were *Salmonella* contaminated after cleaning and disinfection than after unloading the birds. Even more, during this process, the crates may become contaminated with other *Salmonella* serotypes than originally present in the crates (Corry *et al.* 2002). Rigby *et al.* (1980) have shown that the transport of broilers in *Salmonella* contaminated containers led to the contamination of the exterior of the birds.

Mulder (1996) identified catching, loading and transport as stress factors. Transport-induced stress may occur as a result of factors such as crowding, motion, temperature fluctuations and feed and water deprivation. Stressed animals have increased peristaltic movements and excrete pathogenic microorganisms more frequently (Linton and Hinton, 1986). White *et al.* (2001) reported significant increases of *Campylobacter* in broiler ceca following transportation. Consequently, a significant increase in *Campylobacter* contamination of the exterior of the birds after transport was observed (Stern *et al.*, 1995). Therefore, if the

contamination level on the exterior of the birds is extremely high before slaughter, the bacteria on the bird exteriors could contribute to the levels found on fully processed carcasses (Stern *et al.*, 1995).

Just before slaughter, birds are subjected to feed withdrawal during transport to the slaughterhouse. Although the intestines are the primary site of *Campylobacter* and *Salmonella* colonization of poultry, feed withdrawal may influence the crop colonization. Several studies from the same research group (Hagris *et al.*, 1995; Ramirez *et al.*, 1997; Corrier *et al.*, 1999) demonstrated that feed withdrawal in market-age broilers resulted in an increased incidence of *Salmonella*-positive crops and less pronounced in *Salmonella*-positive ceca. The number of *Salmonella*-positive crops may even exceed the number of positive ceca. Byrd *et al.* (1998) showed that following feed withdrawal significant more crops were *Campylobacter* contaminated than ceca.

4.2. Description of a Belgian poultry slaughterhouse

Birds are delivered to the slaughterhouse in transport containers. Modern Belgian slaughterhouses have processing capacities of 6000 to 9000 birds per hour. The live birds are manually hanged by their legs on shackles on a moving line. They are stunned by electrical shock and killed by bleeding. An alternative is to stun the broilers by CO_2 before hanging the birds on the slaughter line. During scalding, the feathers are loosened by submerging the carcasses in a water bath at a temperature of ± 51°C. The feathers are subsequently removed on a plucking machine by means of a series of rotating discs, each with several rubber fingers. The head of the bird and the feet are removed before the carcasses are hung over on a second moving line, the evisceration line. Together with different procedures to remove crop, neck and internal organs, the carcasses are eviscerated mechanically by spoons or clamps. At frequent intervals along the line, water is used to wash both the carcasses and the equipment. The most important washing point is immediately prior to chilling when the carcasses are washed inside and outside. Finally, carcasses are chilled by air-chilling.

A control measure to reduce contamination of carcasses with *Salmonella* is logistic slaughter which is applied in Belgium since 1999. This means that each day *Salmonella*-free flocks are slaughtered first followed by *Salmonella*-positive flocks. The *Salmonella* status is determined by analysis of fecal samples collected in the poultry house maximum three weeks before

slaughter. There are different possibilities to collect these fecal samples: two pairs of overshoes (only for broiler flocks), 60 swabs of fecal material or a pooled sample of 60 fecal samples. At present, no such control measure is implemented for *Campylobacter.*

4.3. Contamination of carcasses during slaughter

Contamination of the carcass by intestinal content during slaughter

Data about *Salmonella* is limited; most recent studies focus on *Campylobacter* which is at present of more importance given the higher flock prevalence. Despite the use of different methods for sampling and quantification, the same observations were made in most studies. Campylobacters are present on the carcasses throughout the whole slaughter process, but the levels may decrease during scalding, chilling and freezing and may increase during defeathering and evisceration (Figure 7).

As explained above, it is likely that there are already a large number of campylobacters on the skin when a broiler enters the processing plant, especially when the flock was already colonized with *Campylobacter*. This is demonstrated by several studies, in which *Campylobacter* have been recovered from broiler carcasses prior to entering the scalding tank (Izat *et al.*, 1988; Berrang and Dickens, 2000; Berrang *et al.*, 2000). Though the numbers present on the exterior of the birds are reduced by scalding, survival of *Campylobacter* has been reported (Oosterom *et al.*, 1983; Izat *et al.*, 1988).

Spilling of the intestinal content of colonized flocks is the most important factor contributing to carcass contamination during slaughter and is difficult to prevent (Oosterom *et al.*, 1983; Berndtson *et al.*, 1992; Ono and Yamamoto, 1999). Berrang *et al.* (2004) have shown that even small amounts (5mg) of intestinal content can cause a significant increase in the numbers of *Campylobacter* on broiler carcasses. During the different stages of the slaughter process, the crop or intestines may be damaged or the content may leach and cause an additional contamination on the carcasses. Feather removal by the mechanical picker may remove bacteria that are associated with the feathers and the skin of the bird (Hinton *et al.*, 2004). On the other hand, the rubber fingers applied in the defeathering process exert pressure on the carcasses, forcing potential contaminated fecal material out and spreading it on the carcasses and the slaughter equipment (Oosterom *et al.*, 1983). Berrang *et al.* (2001) showed

that carcasses plugged with tampons and sutured were significantly less contaminated with campylobacters just after defeathering than control carcasses which were unplugged and unsutured. During evisceration, the intestines can rupture and leak fecal material. Several studies have shown that *Campylobacter* contamination levels increase during the evisceration and decrease during air and water chilling and freezing (Oosterom *et al.*, 1983; Izat *et al.*, 1988; Rosenquist *et al.*, 2006). Despite the fact that water chilling may lead to cross-contamination, this method washes off *Campylobacter* from the surface of the carcasses. The drying effect of air chilling causes physical stress for *Campylobacter*. In the study of Rosenquist *et al.* (2006), the reductions obtained by water and air chilling were very similar, therefore none of the methods could be preferred to the other. Several studies have reported a reducing effect of freezing (Oosterom *et al.*, 1983). Therefore, this technique has been implemented as intervention in broiler processing in Norway, Iceland and Denmark. In practice, this means that *Campylobacter*-positive flocks are used for the production of frozen chicken meat (Rosenquist *et al.*, 2006).

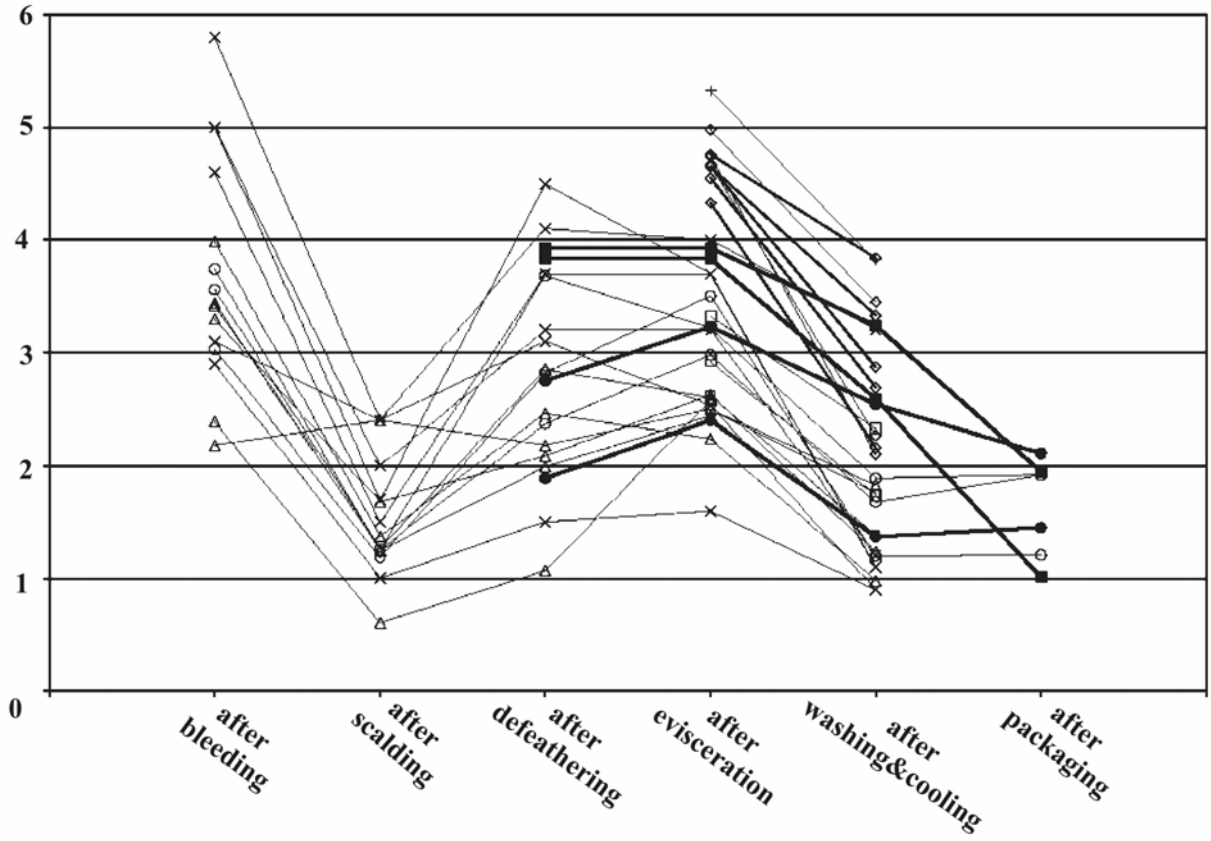

Figure 7. The influence of selected processing operations on the *Campylobacter* contamination of broiler carcasses (in log concentration of bacteria). Δ Oosterom *et al.* (1983b); □ Wempe *et al.* (1983); ○ Izat *et al.* (1988); + Cason *et al.* (1997); × Berrang and Dickens (2000); ◊ Stern and Robach (2003); ● ■ Rosenquist *et al.* (2006). The relative concentration of *Campylobacter* is given in: log10 CFU per g, per 10 000 cm^2, per chicken or per ml carcass rinse (Chart: Rosenquist *et al.*, 2006).

A comparison of the level of *Campylobacter* contamination between different studies is difficult due to the wide variety of methods used, such as examination of an area of the skin (the skin itself or swabs), a certain weight of skin or meat, or whole carcass rinses. Overall, the slaughter process may reduce the level of contamination with 2 or 3 log. Mead *et al.* (1995) found that during processing of broiler flocks colonized with a mean of log 6.8 CFU *Campylobacter* per g of cecal content, there was a reduction in the numbers of campylobacters on the neck skins samples from log 3.7 CFU/g after exsanguinations to log 1.8 CFU/g after slaughter. Izat *et al.* (1988) found a reduction from log 3.5/1000 cm^2 skin on broilers entering the plant to log 1.8/1000 cm^2 skin on carcasses just before package.

Cross-contamination during slaughter

In addition to carcass contamination due to leakage from the crop or intestinal content from the *Campylobacter* or *Salmonella* colonized flock itself, cross-contamination to carcasses from the same flock or other flocks has also been reported. That way, the number of *Campylobacter* or *Salmonella* contaminated carcasses increases during processing. Jones *et al.* (1991a) isolated campylobacters from 20% of the cloacal swabs taken from birds entering the plant and from 52% of the carcasses following immersion chilling. Carramiñana *et al.* (1997) demonstrated the same for *Salmonella*; the *Salmonella* prevalence increased from 30% in fecal material collected from incoming birds to 60% air-chilled carcasses. Lillard (1990) showed that significantly more carcasses were *Salmonella* contaminated exiting the immersion chiller than pre-chilled carcasses. Since there was no increase in *Salmonella* incidence on carcasses from other sampling points starting from pre-scalding to pre-chilling, immersion chilling may be the point of most significant cross-contamination in broiler processing plants in which immersion chilling is used.

Cross-contamination can occur via direct contact between the carcasses, via processing water such as scalding water and chilling water, via the slaughter line, via contaminated hands or knives and via the air. Oosterom *et al.* (1983), Berndtson *et al.* (1996a), and Ono and Yamamoto (1999) isolated campylobacters from the slaughter equipment during processing, such as scalding water, defeathering machine, neck puller, vent cutter, evisceration equipment, conveyer belt, chillers and from the hands of the personnel that came into contact with the carcasses. Genigeorgis *et al.* (1986) demonstrated that *Campylobacter* contaminated

equipment due to poor sanitation at the end of the day (only washed with potable water) may be the source of contamination on broiler carcasses the next day. In other studies, no campylobacters were isolated from the slaughter equipment after cleaning and disinfection (Ono and Yamamoto, 1999; Miwa *et al.*, 2003). In contrast, *Salmonella* was isolated from the slaughter line before processing (Corry *et al.*, 2002; Olsen *et al.*, 2003). Certain *Salmonella* strains were demonstrated to persist on the slaughter line for five days (Olsen *et al.*, 2003).

The excessive use of water during the slaughter process produces a lot of aerosols which may be a vector for horizontal airborne transmission. Posch *et al.* (2006) isolated campylobacters from the aerosols in a poultry slaughterhouse with an average of 3.6×10^3 CFU/m^3 air in the scalding area and 1.3×10^4 CFU/m^3 air in the evisceration area. According to Allen *et al.* (2003), the microbial cross-contamination of broiler chicken carcasses during defeathering occurs mainly via the airborne route.

More recently, molecular tools are used to demonstrate cross-contamination. Miwa *et al.* (2003) demonstrated that *Campylobacter* strains isolated from the carcasses of originally *Campylobacter*-free flocks were the same as those isolated from the intestinal content of previously processed *Campylobacter* colonized flocks. Newell *et al.* (2001) demonstrated that the carcasses of two flocks slaughtered subsequently were contaminated with the same *Campylobacter* strain, which was also isolated from the ceca from the first slaughtered flock. Before the slaughter of the second flock, this strain was also isolated from the scalding tank and the plucking machine. Cross-contamination was also demonstrated by the presence of the same *Campylobacter fla*A type on the carcasses of three successively slaughtered flocks (Rivoal *et al.*, 1999). Olsen *et al.* (2003) demonstrated cross-contamination by *Salmonella* from flocks to the slaughter line and from the slaughter line to flocks which were slaughtered a few days later.

5. IDENTIFICATION AND CHARACTERIZATION

5.1. Campylobacter

Campylobacter jejuni and *C. coli* have been traditionally differentiated by the hippurate hydrolysis test, for which only *C. jejuni* gives a positve reaction. However, hippurate-negative strains of *C. jejuni* are well recognized and problems with false positive test results for non-*C. jejuni* species have also been described (Morris *et al.*, 1985; Denis *et* al., 1999). Nowadays, a wide range of PCR assays are available for the identification of *C. jejuni* and *C. coli*. In the study of On and Jordan (2003), the sensitivity and specificity of 11 PCR assays were evaluated for the identification of these two species. Based on the results of their study, it is recommended to use the multiplex PCR described by Vandamme *et al.* (1997) for concurrent identification and discrimination of *C. jejuni* and *C. coli*, or the PCR assays described by Linton *et al.* (1997).

Although a range of phenotypic methods have been described for typing campylobacters such as biotyping, serotyping and phage typing, these techniques are insufficiently discriminatory and consequently of limited value for epidemiological research. Genotyping methods, such as flagellin gene PCR/restriction fragment length polymorphism (*fla* typing), random amplification of polymorphic DNA (RAPD), multilocus sequence typing (MLST), amplified fragment length polymorphism fingerprinting (AFLP), ribotyping, and pulsed-field gel electrophoresis (PFGE) are currently used for characterizing *Campylobacter* strains. Campynet was a network financed by the European Union for the harmonization and standardization of molecular typing methods for *C. jejuni* and *C. coli*. In this project some key molecular methods were selected for standardization to facilitate epidemiological studies within and between EU countries. Protocols for *fla* typing and PFGE are accessible on the website of Campynet (2001).

Fla typing

The characteristic motility of *C. jejuni* is due to its possession of a polar flagellum at one or both ends of the cell. The flagellar filaments are composed of repeats of a flagellin subunit encoded by two genes (*fla*A and *fla*B). These two flagellin genes are arranged in tandem and

are separated by approximately 170 nucleotides. Because both highly conserved and variable regions are present, these genes are suitable for restriction fragment length polymorphism (RFLP) analysis of a PCR product. The conserved regions in these genes are also partially conserved in species other than *C. jejuni*. *Fla* typing has also proven to be valuable for the majority of *C. coli* strains.

Several *fla* typing procedures have been developed with variations in the primer design, annealing temperatures and restriction enzymes used (Wassenaar and Newell, 2000). In the study of Harrington *et al.* (2003) which was performed as a part of the Campynet network, three of the more commonly used flagellin gene typing assays were compared. On the basis of typeability, inter-laboratory reproducibility and discriminatory abilities, the method of Nachamkin *et al.* (1993) was preferred. This method is based on the amplification of the *fla*A gene alone and *Dde*I as restriction enzyme. When a single enzyme is used, *Dde*I has repeatedly been confirmed as more discriminatory than *Hinf*I, *Pst*I or *Eco*RI while *Alu*I has been found to generate bands too small to be practical for analysis (Wassenaar and Newell, 2000).

It is generally accepted that *fla* typing represents a useful first choice method, as it is cheaper, more widely available, less time-consuming and less labor intensive than other methods (Harrington *et al.*, 2003). However, due to genetic instability as explained below, it should always be used in combination with another molecular characterization method.

PFGE

Digestion of the whole genomic DNA by "rare-cutting" restriction enzymes has proven to be a useful typing technique for many bacteria and is often considered as the "gold standard" (Tenover *et al.*, 1995; Olive and Bean, 1999). Bacterial cells are embedded in agarose and lysed *in situ* to prevent DNA shearing. After several washing steps to remove chemicals, thin slices of the DNA containing blocks are cut and the restriction enzyme of choice is applied. The aim is to cut the DNA into a few, comparatively large fragments which can be separated by using electrophoretic conditions under which the orientation of the electrical field is changed in a pulsed manner.

PFGE is generally accepted as one of the most powerful tools currently available. The sensitivity of this technique lies in the fact that whole-genome restriction site polymorphisms are detected. Comparisons of the discriminatory power of PFGE with other molecular methods for typing *C. jejuni* and *C. coli* show that PFGE is extremely sensitive. Only AFLP appears to equal its discriminatory potential (Kokotovic and On, 1999). As for *fla* typing, the protocols used for PFGE in different studies vary; differences in electrophoretic conditions and restriction enzymes lead to differences in profiles. Both Campynet (2001) and PulseNet (2006) proposed standard methods for PFGE typing of campylobacters to make comparisons of the PFGE profiles obtained by different laboratories possible. In their protocol *Sma*I was proposed as restriction enzyme, though satisfactory results have also been obtained with *Kpn*I, *Sal*I, *Apa*I and *Bss*HII (Wassenaar and Newell, 2000).

Genetic instability

For successful application of a typing method, inherent stability of the subtype marker is essential. One of the major disadvantages of *fla* typing is related to genetic instability. Harrington *et al.* (1997) provided evidence for intergenomic recombination between *fla*A genes of different strains. Moreover, there was evidence for intragenomic recombination between the *fla*A and *fla*B genes of individual strains. So far, *fla* genotypes have proven to be stable during freezing and storage and are probably stable during short-term outbreaks, such as poultry flock infections (Wassenaar and Newell, 2000). Nevertheless, the possibility that genotypic instability occurs in response to environmental pressures cannot be ignored. Therefore, Wassenaar and Newell (2000) have recommended combining *fla* typing with another genotypic method in order to identify changes in a given *fla* type.

As the genome of *Campylobacter* may ondergo mosaic rearrangement on genomic scale, other genotypic methods may also be influenced by genetic instability. On (1998) demonstrated that long-term subcultering (50 times over a 6-month period) can result in changed PFGE profiles. Other studies found *fla* typing to be stable, whereas PFGE types showed considerable genomic rearrangement (Wassenaar *et al.*, 1998; De Boer *et al.*, 2002). For this reasons, it is recommended to use a combination of typing methods (Wassenaar and Newell, 2000) or to use molecular techniques which are less sensitive to changes in the

genome. RAPD and AFLP generate small bands and are therefore the least sensitive to genetic instability.

5.2. Salmonella

Traditionally, epidemiological investigations for *Salmonella enterica* have been based on phenotypic characteristics such as serotyping, phage typing and patterns of resistance to antimicrobials. The internationally used method for characterizing *Salmonella* is serotyping of the isolates according to the Kauffmann-White scheme (Popoff and Le Minor, 1997). According to this scheme, each *Salmonella* serotype is recognized by its possession of particular lipopolysaccharides, or O antigens and flagellar proteins, or H antigens. However, serotyping is often restricted to national reference laboratories, expensive and time-consuming. In contrast, PCR-based methods are available in a lot of laboratories, easy to perform, rapid and relatively cheap. Therefore, several attempts have been made to correlate serotypes with genotypes (Van Lith and Aarts, 1994; Milleman *et al.,* 1996; Burr *et al.,* 1998; Johnson and Clabots, 2000; Johnson *et al.*, 2001). These studies yielded conflicting results, and at the moment such correlation technique is not available.

Molecular characterization methods are plasmid profiling, pulsed-field gel electrophoresis (PFGE) and PCR-based techniques such as restriction fragment length polymorphism (PCR-RFLP), random amplification of polymorphic DNA (RAPD), multilocus sequence typing (MLST), amplified fragment length polymorphism fingerprinting (AFLP) and repetitive extragenic palindromic PCR (rep-PCR). Since each technique has his advantages and disadvantages, there is at present no consensus about which methods are best suited for intraserotype differentiation in *Salmonella enterica* (Liebana, 2002)

PFGE

As mentioned earlier, PFGE is generally considered as the gold standard. Many epidemiological typing studies have successfully applied this method as a basis for identification of strains in *Salmonella enterica*. PFGE has been proven to be a good discriminating tool for typing of *Salmonella* Typhimurium, *Salmonella* Dublin, *Salmonella* Infantis, *Salmonella* Virchow and *Salmonella* Hadar (Liebana, 2002). In spite of this, some

studies have reported the limitations of PFGE for differentiating *Salmonella* Enteritidis isolates (Thong *et al.*, 1995; Liebana *et al.*, 2001). Therefore, it is recommended to use a combination of different restriction ezymes and/or other genotypic techniques for discrimination (Liebana, 2002).

At the moment, PFGE is the preferred and the only subtyping method of PulseNet. This network was developed in 1996 by the Center for Disease Control and Prevention for the detection and investigation of outbreaks of foodborne infections in the US. In this network, standardized PFGE methods have been developed so that scientists at public health laboratories can rapidly compare PFGE patterns of certain foodborne bacteria. These standardized PFGE protocols are available online (PulseNet, 2006).

AIMS OF THE STUDY

Salmonella and *Campylobacter* are two important bacterial causes of gastroenteritis in the industrialized world. Poultry meat is considered to be an important source in the transmission to humans for both zoonoses. Most studies focus on the farm level to reduce the number of flocks colonized with these zoonoses in order to lower the risk of infection for humans. However, transport and processing of poultry are as important since *Salmonella* and *Campylobacter* can be spread during these last steps leading to the contamination of the end product.

The general aim of this thesis was to study the *Salmonella* and *Campylobacter* contamination of poultry during transport and slaughter by means of molecular tools.

The specific aims of this thesis were as follows:

- to compare rep-PCR based methods for molecular discrimination of *Salmonella* serotypes (Chapter I)
- to study the contribution of gastrointestinal colonization and cross-contamination to carcass contamination during poultry slaughter for *Salmonella* and *Campylobacter* (Chapter II and III)
- to investigate an association between colonization of poultry flocks with *Salmonella* and *Campylobacter* and to evaluate the best sampling site for determining the prevalence of these two pathogens in flocks at slaughterhouse level (Chapter IV)
- to determine the impact of *Salmonella* present on the slaughter line on carcass contamination (Chapter V)
- to determine the impact of *Campylobacter* present in transport containers on the contamination/colonization of transported flocks (Chapter VI)

CHAPTER I

Comparison of five repetitive-sequence-based PCR typing methods for molecular discrimination of *Salmonella enterica* isolates

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Rasschaert G., K. Houf, H. Imberechts, K. Grijspeerdt, L. De Zutter, M. Heyndrickx (2005). Comparison of five repetitive-sequence-based PCR typing methods for molecular discrimination of *Salmonella enterica* isolates. *J. Clin. Microbiol.* 43: 3615–3623.

Summary

Five repetitive-element PCR (rep-PCR) techniques [primer sets ERIC1R-ERIC2 and REP1R-REP2I and primers ERIC2, BOXA1R and $(GTG)_5$] were evaluated for the discrimination of *Salmonella enterica* isolates at the serotype level. On the basis of number, even distribution over the whole fingerprints and clarity of bands in the fingerprints, the enterobacterial repetitive intergenic consensus (ERIC) primer set and the $(GTG)_5$ primer were chosen for use in the following experiments. For these two primer sets, reproducibility was tested on different lysates of five selected serotypes of *Salmonella* in the same PCR by using three different PCR runs. Reproducibility was poor between different PCR runs but high within the same PCR run. Furthermore, 80 different serotypes and five isolates which were not typeable by serotyping were fingerprinted. All strains were typeable by the ERIC primer set and the $(GTG)_5$ primer and generated unique fingerprints, except for some strains with incomplete antigenic codes. Finally, 55 genetically different strains belonging to 10 serotypes were fingerprinted to examine the genetic diversity of the rep-PCR within serotypes. This experiment showed that one serotype did not always correlate to only one ERIC or $(GTG)_5$ fingerprint but that the fingerprint heterogeneity within a serotype was limited. In epidemiological studies, ERIC- and/or $(GTG)_5$-PCR can be used to limit the number of strains that has to be serotyped. The reproducibility of isolates in one PCR run, the discriminatory power and the genetic diversity (stability) of the fingerprint were similar for the ERIC primer set and the $(GTG)_5$ primer, so both primers are equally able to discriminate *Salmonella* serotypes.

1. INTRODUCTION

Salmonella enterica is one of the major causes of human gastroenteritis worldwide. In Belgium, 12 894 human *Salmonella* isolates were received in 2003 by the National Reference Centre for *Salmonella* and *Shigella* (NRSS, 2003). The most common serotypes isolated were *Salmonella* serotype Enteritidis, *Salmonella* serotype Typhimurium, *Salmonella* serotype Virchow, *Salmonella* serotype Derby and *Salmonella* serotype Brandenburg. The internationally used method for characterizing *Salmonella* is serotyping of the isolates according to the Kauffmann-White scheme (Popoff and Le Minor, 1997). Each *Salmonella* serotype is characterized by the combined expression of particular lipopolysaccharides, or O antigens and flagellar proteins, or H antigens. Currently, more than 2500 serotypes are recognized (Popoff *et al.*, 2004). In most countries, serotyping is restricted to national reference laboratories, to which clinical and food microbiology laboratories send *Salmonella* isolates. Serotyping, especially of less abundant types, is expensive and time-consuming. In addition, according to the National Reference Centre for *Salmonella* and *Shigella* (NRSS, 2003) and the Belgian Reference Laboratory for *Salmonella* (2003), respectively, 0.12% of the human isolates and 4% of the isolates from animal origin were not typeable in 2003 (by autoagglutination).

PCR-based molecular techniques are easy to perform and rapid. Repetitive-element PCR (rep-PCR) uses primers complementary to naturally occurring, highly conserved, repetitive DNA sequences. These noncoding sequences are present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria (Lupski and Weinstock, 1992). Examples of these repetitive elements are the repetitive extragenic palindromic (REP) sequences, the enterobacterial repetitive intergenic consensus (ERIC) sequences, the BOX sequences and the polytrinucleotide $(GTG)_5$ sequence (Versalovic *et al.*, 1994).

According to Van Lith and Aarts (1994), it is possible to use the primer set ERIC1R-ERIC2 to discriminate *Salmonella* serotypes. Burr *et al.* (1998) and Milleman *et al.* (1996) tested the same primer set and concluded that the obtained fingerprints were not correlated with serotypes. Two studies (Johnson and Clabots, 2000; Johnson *et al.*, 2001) showed that elevated annealing temperatures combined with the use of a commercial PCR mix improve the reproducibility and the resolving power of rep-PCR with the ERIC2 and BOXA1R primers.

The studies performed yielded conflicting results and evaluated only the ERIC primer set, the ERIC2 primer and/or the BOX primer on a limited number of serotypes and/or on a limited number of strains per serotype. The purpose of this study was to evaluate five different rep-PCR techniques for the discrimination of *Salmonella* isolates including the $(GTG)_5$ primer (Versalovic *et al.*, 1994; Gevers *et al.*, 2001). Selected rep-PCR techniques were further evaluated for their powers of discriminating between as many as 80 different serotypes as well as for the genetic diversity within several serotypes.

2. MATERIALS AND METHODS

***Salmonella* isolates.** The *Salmonella* isolates were isolated from the following sources: human feces, poultry, eggs, pigeons, cattle, swine, deer, reptiles, water, farm environments and farm equipment. Almost all isolates were isolated in Belgium from the year 1998 until 2003 (Table 1). They were serotyped at the Belgian *Salmonella* reference laboratories according to the Kauffmann-White scheme (Popoff and Le Minor, 1997).

DNA isolation. The bacteria were grown overnight on tryptone soy agar plates (TSA, Oxoid CM0131, Basingstoke, UK) at 37°C. The cells were harvested and resuspended in 300 µl 0.05 M NaOH-0.125% (wt/vol) sodium dodecyl sulfate and heated at 90°C for 17 min. The lysates were stored at -20°C until use, which was approximately 3 weeks later. The lysates were used only once. Before use in the PCR, the lysates were centrifuged for 2 min at 10 000 rpm.

Only when elevated annealing temperatures were tested as described by Johnson and Clabots (2000) were DNA extracts used instead of lysates. DNA was extracted using a commercial genomic DNA purification kit (AquaPure genomic DNA isolation kit 732-6340; Bio-Rad Laboratories, Hercules, California, USA) according to the manufacturer's instructions.

Selection of primers for rep-PCR. *Salmonella* strains belonging to 22 serotypes were typed with the primer sets ERIC1R-ERIC2 and REP1R-REP2I and primers ERIC2, BOXA1R and $(GTG)_5$. The best primer or primer set and the best corresponding annealing temperatures were selected on the basis of the number, distribution and clarity of bands in the obtained fingerprints.

Reproducibility of rep-PCR. In the second experiment, three different lysates were made on three separate days, starting from different bacterial cultures of five selected serotypes (Figure 1). Reproducibility was evaluated on the different lysates by using three different PCR runs on the same thermal cycler.

Rep-PCR on isolates belonging to different serotypes. Eighty serotypes and five isolates which were not typeable by serotyping were characterized to test the typeability and the discriminatory power of the selected primer sets.

Rep-PCR on isolates belonging to the same serotypes. Strains belonging to the same serotype, but of different origins and genetically different, were fingerprinted to examine the genetic diversity (stability) of the rep-PCR within serotypes with the selected primer or primer set. The aim of this experiment was to investigate whether genetically different strains of the same serotype resulted in the same fingerprint. The genetic diversity of the strains had been tested by pulsed-field gel electrophoresis using *Xba*I as the restriction enzyme (Botteldoorn *et al.*, 2004). Fifty-five strains from 10 different serotypes were fingerprinted: 13 serotype Enteritidis, 9 serotype Typhimurium, 5 serotype Hadar, 5 serotype Derby, 5 serotype Virchow, 5 serotype Infantis, 4 serotype Blockley, 4 serotype Brandenburg, 3 serotype Agona and 2 serotype Indiana strains.

PCR. PCR amplifications were performed in a Perkin-Elmer 9700. The sequence of the primers used and the amplification protocols were those described by Versalovic *et al.* (1994). The reaction mixtures for primers ERIC1R, ERIC2, REP1R, REP2I and BOXA1R were as described by Rademaker and de Bruijn (1997), but Tween20 (0.5%) and gelatin (0.01%) (Heyndrickx *et al.*, 2002) were added when lysates were used. The reaction mix for the $(GTG)_5$ primer contained the following: 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 60 pmol $(GTG)_5$ (Eurogentec, Seraing, Belgium), 0.5% (vol/vol) Tween 20, 0.01% (wt/vol) gelatin, 1 U *Taq* DNA polymerase (YellowStar Taq, Eurogentec, Seraing, Belgium) and 1 µl crude cell lysate with a final volume of 25 µl.

Elevated annealing temperatures were also tested in the first experiment. An annealing temperature of 70°C for ERIC2 and BOXA1R and an amplification protocol of 35 cycles without an initial touch-down were used (Johnson and Clabots, 2000) but with the reaction mix of Rademaker and de Bruijn (1997) instead of Ready to Go PCR beads (Johnson and

Clabots, 2000). Elevated annealing temperatures were also tested using modified amplification protocols of Versalovic *et al.* (1994). Modifications consisted of annealing temperatures of 57°C for ERIC2 and 65°C for BOXA1R.

The PCR products were size separated in a 1.5% agarose gel in 1 x Tris-borate-EDTA at 120 V for 4 h. The gels were stained with ethidium bromide and digitally captured under UV light. The gel images were visually compared and analyzed with GelCompar, version 3.0 (Applied Maths, Kortrijk, Belgium) using a mixture of a 100-bp (Invitrogen, Paisley, UK) and a 500-bp ladder (Bio-Rad Laboratories, Hercules, California, USA) as the normalization reference (Rademaker and de Bruijn, 1997). The similarities between the fingerprints were calculated using the Pearson correlation (with an optimization of 1% and a position tolerance of 1%) and the fingerprints were grouped according to their similarities by use of the UPGMA (unweighted-pair group method using arithmetic averages) algorithm.

Statistical analysis of dendrograms. To assess the variability introduced by the preparation of lysates and the PCR run in the clustering of the fingerprints, several indices were derived from the similarity matrix obtained from experiment 2 (reproducibility of rep-PCR) by the method of Johnson and Clabots (2000). For all strains, a similarity index (SI) was calculated as the mean of all pairwise correlations between different replicates of a strain at a certain level of a factor (lysate or PCR-run). A lower SI can be interpreted as more variability attributable to the factor. The differentiation index (DI) was defined as the maximum of all pairwise correlations between different strains, at a certain level of a factor in one strain and all possible combinations in the other; this was repeated for each strain. A higher DI can be interpreted as more variability attributable to the factor. Finally, the difference between the similarity index and the differentiation index (called ‘net discriminating power’ by Johnson and Clabots (2000)) can be considered to be a measure for the discriminatory power of the strain. A higher value, calculated as 100-(SI-DI), which can be defined as the net variability index, means more variability attributable to the factor on the clustering.

Table 1. *Salmonella* isolates used in the experiments

serotype	isolate n°	source	serotype	isolate n°	source
S. Aba	VVG03/0027	pork	*S.* Kedougou[b]	MB1433	egg white powder
S. Aberdeen	MB2555	human	*S.* Kentucky	KS192	overshoes in poultry house
S. Adelaide[a]	MB526	not known	*S.* Kiambu	VVG00/1507	poultry meat
S. Agona	KS002	broiler's feces	*S.* Kingstone	VVGN077	mesenteric lymph node pig
S. Agona	MB1230	poultry meat	*S.* Kintambo	MB2507	surface isolation
S. Agona	MB2506	industrial surface	*S.* Kottbus	MB2546	human
S. Agona	5.22RV	broiler's carcass	*S.* Larochelle	MB2544	human
S. Altona	MB2549	human	*S.* Litchfield	MB2548	not known
S. Anatum	KS187	overshoes in poultry house	*S.* Livingstone	VVG01/0925	pork
S. Apapa	MB2560	not known	*S.* London	VVG02/0819	pork
S. Bareilly	MB1253	not known	*S.* Manhattan	MB1260	not known
S. Blockley	3.1D	broiler's feces	*S.* Mbandaka	KS61	environment of poultry house
S. Blockley	10.4E	broiler's feces	*S.* Meleagridis	VVG00/1923	pig
S. Blockley	KS109	feed in broiler house	S. Minnesota	MB2558	human
S. Blockley	KS163	overshoes in poultry house	*S.* Montevideo	VVG13.20K	broiler's feces
S. Blockley	VVG02/703	undetermined food	*S.* Muenchen	MB2547	not known
S. Bovismorbificans	VVG02/1518	pig's carcass	*S.* Muenster	VVGN049	mesenteric lymph node pig
S. Braenderup	KS113	overshoes in poultry house	*S.* Newport	MB1246	not known
S. Brandenburg	KS181	cecal dropping of broiler	*S.* Ohio	VVG01/0822	pig's carcass
S. Brandenburg	MB1720	pig slaughterhouse	*S.* Oranienburg	VVGN179	mesenteric lymph node pig
S. Brandenburg	MB1722	pig slaughterhouse	*S.* Panama	VVG02/0923	pork
S. Brandenburg	MB1724	pig slaughterhouse	*S.* Paratyphi A	MB2541	human
S. Brandenburg	VVG02/0928	pork	*S.* Paratyphi B	VVG02/0726	beef
S. Bredeney	VVG02/0784	chicken filet	*S.* Plymouth	MB2553	human
S. Cerro	MB2368	environment food factory	*S.* Poona	VS821475c	mesenteric lymph node pig
S. Chester	MB2543	not known	*S.* Pullorum	MB2349	poultry
S. Coeln	MB1080	not known	*S.* Putten	VVGP109M	feathers during plucking process of broiler
S. Concord	VVG03/0546	human	*S.* Rissen	VVGK015	poultry
S. Derby	MB1531	pig	*S.* Rubislaw	MB2556	not known
S. Derby	MB1736	pig's carcass	*S.* Sandiego	VVG02/0235	undetermined food
S. Derby	MB1737	pig slaughterhouse	*S.* Senftenberg	MB1559	feed from broiler house
S. Derby	MB1739	pig	*S.* Stanleyville	MB1312	egg
S. Derby	MB1745	pig slaughterhouse	*S.* Stourbridge	MB2550	human
S. Derby	VVG02/1145	pork	*S.* Sundsvall	VVGN485	mesenteric lymph node pig
S. Dublin	VVG01Z/1018	cattle's carcass	*S.* Swartzengrund	VS112916c	pig's feed trough
S. Enteritidis	KS104	egg	*S.* Telelkebir	MB2557	not known
S. Enteritidis	KS157	overshoes in poultry house	*S.* Tennessee	MB1198	poultry feed
S. Enteritidis	KS585	paper tray liners (transport)	*S.* Thompson	VVG01/0010	poultry meat
S. Enteritidis	MB1208	human feces	*S.* Typhimurium O5-	MB1217	human
S. Enteritidis	MB1221	tiramisu	*S.* Typhimurium O5-	MB1780	pigeon
S. Enteritidis	MB1409	egg	*S.* Typhimurium O5-	MB1786	pigeon
S. Enteritidis[c]	MB1419	egg	*S.* Typhimurium O5-	VVG01/0922	pig
S. Enteritidis[c]	MB1420	egg	*S.* Typhimurium O5+	MB1241	not known
S. Enteritidis[b]	MB1432	egg white powder	*S.* Typhimurium O5+	MB2177	pig carcass
S. Enteritidis[d]	MB1450	human case	*S.* Typhimurium O5+	MB2199	overshoe pig farm
S. Enteritidis	MB1535	deer	*S.* Typhimurium O5+	MB2249	human
S. Enteritidis	MB1677	human	*S.* Typhimurium O5+	MB2274	human
S. Enteritidis	MB2350	chicken	*S.* Typhimurium O5+	MB2299	human

serotype	isolate n°	source	serotype	isolate n°	source
S. Gallinarum	MB2499	reptile	*S.* Urbana	VVGN198	mesenteric lymph node pig
S. Give	VVG01/0873	pig's carcass	*S.* Virchow	KS087	equipment in broiler house
S. Goldcoast	VVG01/0646	pork	*S.* Virchow	MB2339	broilers
S. Hadar	KS077	cecal dropping of broiler	*S.* Virchow	MB2341	chicken filet
S. Hadar	KS106	feed tray in poultry house	*S.* Virchow	MB2342	chicken filet
S. Hadar	MB1134	poultry breeder animals	*S.* Virchow	MB2396	not known
S. Hadar	MB1148	pig	*S.* Virchow	VVG02/1504	poultry meat
S. Hadar	MB1149	poultry	*S.* Waycross	MB2559	human
S. Hadar	VVG02/0777	poultry meat	*S.* Wien	VVG02/0527	undetermined food
S. Havana	VS219353a	pig	*S.* 4:i:-	VVGN385	mesenteric lymph node pig
S. Idikan	MB1235	not known	*S.* 47:z4z23:-	VS219188a	pig
S. Indiana	6.4	broiler's feces	*S.* 6,7:-:5	5.60RV	broiler's carcass
S. Indiana	5.35	broiler's carcass	*S.* 6,7:r:-	MB2529	industrial surface
S. Indiana	VVG02/0038	poultry meat	*S.* 6,8:-:1,2	VVG00/0669	pig's carcass
S. Infantis	KS001	equipment in broiler house	*S.* 9:-:-	MB2551	not known
S. Infantis	MB1146	feed	*S.* IV48:g,z51:-	MB2561	not known
S. Infantis	MB1729	pig slaughterhouse	Not typeable	VVG02/0042	pork
S. Infantis	MB1730	pig slaughterhouse	Not typeable	KS128	overshoes in poultry house
S. Infantis	MB1735	overshoes pig	Not typeable	MB2562	not known
S. Infantis	VVG02/0398	beef	Not typeable	MB2563	not known
S. Isangi	MB1092	not known	Not typeable	MB2564	not known

[a] : originates from Slovakia
[b] : originates from The Netherlands
[c] : originates from Austria
[d] : originates from the UK

3. RESULTS

Selection of primers and annealing temperature. With the ERIC1R-ERIC2 primer set, profiles consisted of 13 to 22 bands, evenly distributed over the entire fingerprint. With the REP1R-REP2I primer set, 5 to 10 bands were obtained for each fingerprint, but most of the bands were weak (data not shown). When the $(GTG)_5$ primer was applied, 11 to 16 bands were visible for each fingerprint, most of them located between 1000 and 2500 bp. With the BOXA1R primer, the fingerprints consisted of more than 25 bands, which made visual comparison between fingerprints very difficult (data not shown). Elevated annealing temperatures of 70°C did not generate any bands for the ERIC2 primer and generated 5 bands, all lower than 500 bp, for the BOXA1R primer (data not shown). Annealing temperatures of 57°C for ERIC2 and 65°C for BOXA1R resulted in fingerprints with 10 to 16 bands and 10 to 14 bands, respectively (data not shown).

According to Versalovic *et al.* (1994), the optimal number of bands for rep-PCR is 8 to 15. The upper limit of bands, however, depends on the resolution of the electrophoretic system; the higher the resolution, the more bands can be reliably separated and visualized. With a 1.5% agarose gel separation on 20-cm-long gels, as used in this study, the upper limit was judged to be around 20 distinct fragments. On the basis of the number and clarity of the bands, their even distribution over the whole fingerprint and discriminatory power, the ERIC primer set and the $(GTG)_5$ primer were chosen for use in subsequent experiments. The above data indicate that this primer and primer set have the greatest potential to discriminate *Salmonella* strains belonging to different serotypes.

Reproducibility. All fingerprints obtained with the ERIC primer set had a band at 250 bp (Figure 1). This high-intensity band was excluded from the calculation of the Pearson correlation in this and subsequent experiments. The Pearson correlation takes the whole profile into account, so exclusion of dense bands often allows more meaningful clustering or groups (Costas, 1992; Heyndrickx *et al.*, 1996).

Each of the five serotypes clustered together with a minimum similarity coefficient of 74% with the ERIC primer set (data not shown), 83% with the $(GTG)_5$ primer (data not shown), and 80% for the composite dataset [(ERIC plus $(GTG)_5$)] (Figure 1). The low similarity coefficients were the result of the three different PCR runs. In Table 2 a quantitative assessment is made of the variability attributable to the PCR run and to the preparation of the lysate in the clustering of the combined ERIC- and $(GTG)_5$-PCR fingerprints. These results clearly indicate that the variability attributable to the PCR run (with the lysate factor kept constant) is systematically higher; this can be deduced from the lower SI values and the higher net variability index [100-(SI-DI)]. It is therefore advisable to compare results within the same PCR run. Within one PCR run, the minimum similarity coefficient between the three different lysates (i.e., lysates made on different days) was 95% for the ERIC primer set, except for four out of a total of 45 lysates (data not shown). The minimum similarity coefficient within one PCR run for the three different lysates was 94% for the $(GTG)_5$ primer, with the exception of six lysates (data not shown). These exceptions were due to overall weaker patterns, locally weaker bands or normalization errors. For the composite data set, the minimum similarity coefficient for the three different lysates within one PCR run was 92.5%, except for two lysates: KS077, made on the first day and processed in the first PCR run, and MB1720, made on the third day and processed in the third PCR run (Figure 1). Based on

these results, the delineation levels for serotype discrimination in subsequent experiments were set at 95% for the ERIC primer set, 94% for the $(GTG)_5$ primer, and 92.5% for the composite data set.

Table 2. Assessment of variability attributable to PCR and lysates in the clustering of rep-PCR fingerprints.

variable factor	constant factor	SI (%)[a]	DI (%)[a]	100-(SI-DI) (%)[a]
PCR run	lysate t1[b]	85.92	61.62	75.70
	lysate t2[b]	87.66	63.14	75.48
	lysate t3[b]	86.10	62.62	76.52
lysate	PCR-run 1[c]	94.34	57.11	62.77
	PCR-run 2[c]	97.12	61.51	64.39
	PCR-run 3[c]	95.76	63.48	67.72

[a]: indices were calculated based on the similarity matrix of the dendrogram of replicate combined ERIC- and $(GTG)_5$-PCR fingerprints of 5 strains as shown in Figure 1. The means over the five strains of the different indices are shown.
[b]: measures variability attributable to three different PCR runs on the same lysate.
[c]: measures variability attributable to three different lysates in the same PCR run.

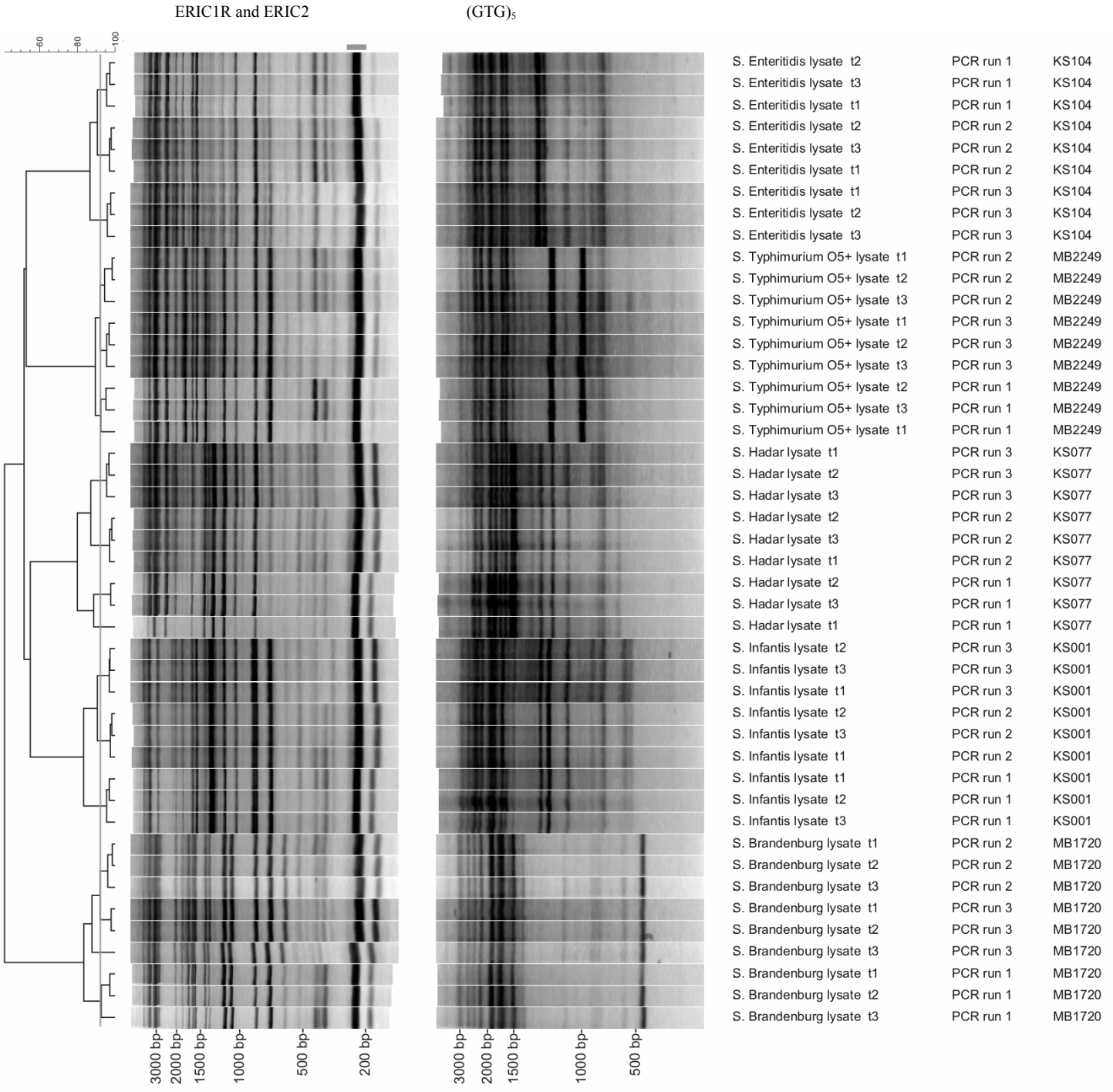


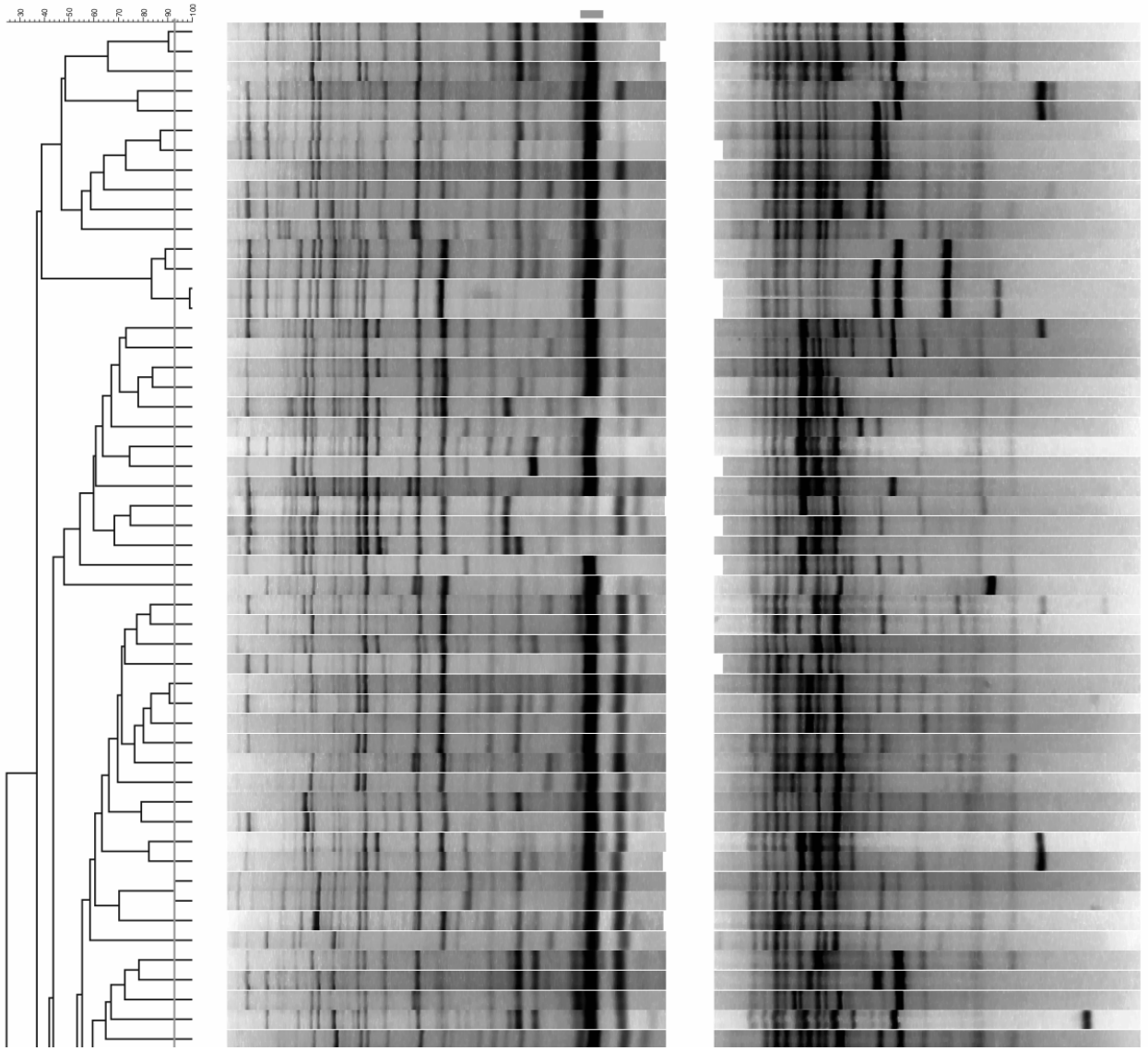
Figure 1. Cluster of the composite data set and ERIC and $(GTG)_5$ fingerprints of three different lysates made on three different days (t1, t2 and t3) and run in three different PCR runs in the same thermal cycler. The similarities between the fingerprints were calculated using the Pearson correlation (optimization, 1%; position tolerance, 1%), and the fingerprints were grouped according to their similarities by use of the UPGMA algorithm. The vertical grey line shows the delineation level of 92.5%. The last column shows the strain numbers. The grey bar at the top of the figure shows the part (79.3% to 84.3%) of the ERIC fingerprints that is not taken into account to calculate the cluster, as explained in the text.

Typeability and discriminatory power. Figure 2 shows the fingerprints of the 80 different serotypes and the five isolates which were not typeable by serotyping, obtained with the ERIC primer set and the $(GTG)_5$ primer and the clustering of the composite data set. Six of the 85 fingerprints obtained with the ERIC primer set, displayed no band at 250 bp. This band was excluded from the calculation of the Pearson correlation, as already mentioned. At a delineation level of 95% for the ERIC primer set, all serotypes generated unique fingerprints except for three pairs of two strains. Two strains (MB2563 and MB2564) which were not typeable by serotyping had a similarity coefficient of 98.8%. Serotype Typhimurium (MB2249) and serotype Typhimurium var. Copenhagen (VVG01/0922) had a similarity coefficient of 96.3%. Serotype Infantis (VVG02/0398) and strain MB2529, with antigenic formula 6,7:r:- had a similarity of 95.6% (data not shown).

At a delineation level of 94% for the $(GTG)_5$ primer, all but nine strains had unique fingerprints. Strains MB2563 and MB2564, which were not typeable by serotyping, had a similarity coefficient of 99.2%. Serotype Enteritidis (MB1409), strain MB2562, which was not typeable by serotyping and strain MB2551 with antigenic formula 9:-:-, had a similarity coefficient of 95%. Two other strains, one serotyped as serotype Paratyphi B (VVG02/0726) and strain KS128, which was not typeable by serotyping, had a similarity coeffient of 96.6%. Serotype Urbana (VVGN198) and serotype Sundsvall (VVGN485) had a similarity coefficient of 94.8%, although visually there was a difference of two bands (data not shown).

In the composite dataset (Figure 2), two pairs of strains were not discriminated from each other at the delineation level of 92.5%. The two nontypeable strains MB2563 and MB2564 had a similarity coefficient of 98.9%, and serotype Paratyphi B (VVG02/0726) and isolate KS128, which was not typeable by serotyping, had a similarity coefficient of 92.9%.

ERIC1R and ERIC2 $(GTG)_5$



S. 6,8 : - : 1,2	6,8 : - : 1,2	.VVG00/0669
S. Muenchen	6,8 : d : 1,2	.MB2547
S. Adelaide	35 : f,g : -	.MB526
S. Gallinarum	1,9,12 : - : -	.MB2350
S. Pullorum	1,9,12 : - : -	.MB2349
S. 9 : - : -	9 : - :-	.MB2551
Not typable		.MB2562
S. Enteritidis	1,9,12 : [f],g,m, [p] : [1,7]	.MB1409
S. London	3,10[15] : l,v : 1,6	.VVG02/0819
S. Manhattan	6,8 : d : 1,5	.MB1260
S. Stanleyville	1,4, [5], 12, 27 : z4z23 : [1,2]	.MB1312
S. Typhimurium	1,4, [5],12 : i : 1,2	.MB2249
S. Typhimurium var. Co.		.VVG01/0922
Not typable		.MB2563
Not typable		.MB2564
S. Panama	1,9,12 : l,v : 1,5	.VVG02/0923
S. Poona	1,13,22 : z : 1,6	.VS821475c
S. Oranienburg	6,7,14 : m,t : [z57]	.VVGN179
S. Urbana	30 : b : e,n,x	.VVGN198
S. Montevideo	6,7,14 : g,m, [p],s : [1,2,7]	.VVG13.20K
S. Wien	1,4,12,27 : b : l,w	.VVG02/0527
S. Bredeney	1,4,12,27 : l,v : 1,7	.VVG02/0784
S. Minnesota	21 : b : e,n,x	.MB2558
S. Give	3,10[15][15,34] : [d],l,v : 1,7	.VVG01/0873
S. Sandiego	4,[5], 12 : e,h : e,n,z15	.VVG02/0235
S. Waycross	41 : z4z23 : -	.MB2559
S. Sundsvall	[1],6,14,[25] : z : e,n,x	.VVGN485
S. Telelkebir	13,23:d:e,n,z15	.MB2557
S. Virchow	6,7 : r : 1,2	.VVG02/1504
S. Aba	6,8 : i : e,n,z15	.VVG03/0027
S. Agona	1,4,[5],12 : f,g,s : [1,2]	.5.22RV
S. Muenster	3,10[15][15,34] : e,h : 1,5	.VVGN049
S. Apapa	45 : m,t : -	.MB2560
S. Derby	1,4, [5],12 : f,g : [1,2]	.VVG02/1145
Not typable		.VVG02/0042
S. Kentucky	8,20 : i : z6	.KS192
S. Kedougou	1,13,23 : i : l,w	.MB1433
S. Concord	6,7 : l,v : 1,2	.VVG03/0546
S. Mbandaka	6,7,14 : z10 : e,n,z15	.KS61
S. 4 : i : -	4 : i : -	.VVGN385
S. Stourbridge	6,8 : b : 1,6	.MB2550
S. Brandenburg	1,4,[5],12,27 : l,v : e, n, z15	.VVG02/0928
S. Chester	1,4,[5],12 : e,h : e,n,x	.MB2543
S. Paratyphi B	1,4, [5], 12 : b : [1,2]	.VVG02/0726
Not typable		.KS128
S. Havana	1,13,23 : f,g,[s] : -	.VS219353a
S. Isangi	6,7,14 : d : 1,5	.MB1092
S. Coeln	4,[5],12 : y : 1,2	.MB1080
S. Dublin	1,9,12,[Vi] : g,p : -	.VVG01/1018
S. Newport	6,8,20 : e,h : 1,2	.MB1246
S. 47 : z4z23 : -	47 : z4z23 : -	.VS219188a
S. Kingston	1,4, [5],12,27 : g,s,t : [1,2]	.VVGN077

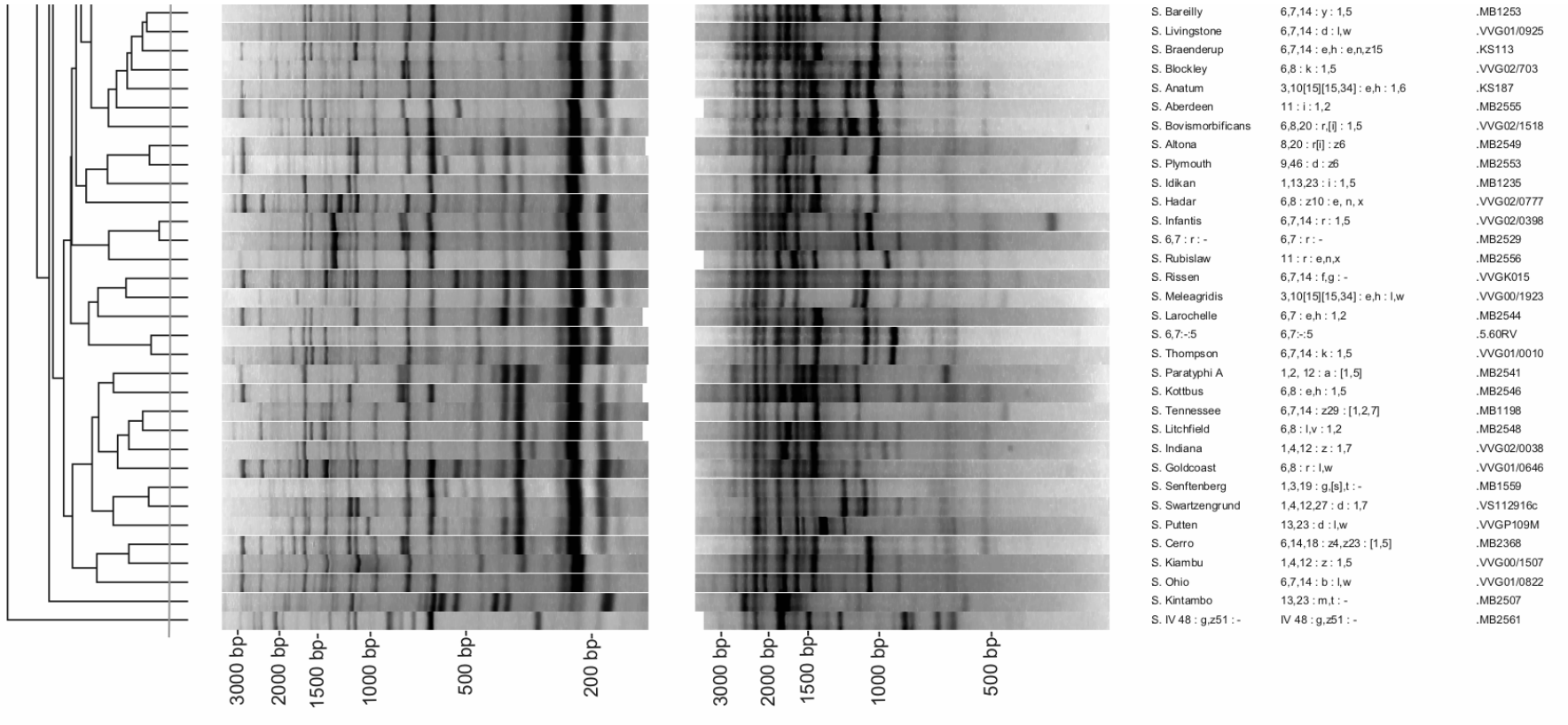


Figure 2. Cluster of the composite data set and ERIC and $(GTG)_5$ fingerprints of 80 different serotypes and five isolates that were not typeable by serotyping. The similarities between the fingerprints were calculated using the Pearson correlation (optimization, 1%; position tolerance, 1%), and the fingerprints were grouped according to their similarities by use of the UPGMA algorithm. The vertical grey line shows the delineation level of 92.5%. The second column shows the antigenic formulas of the serotypes given in the first column. The last column shows the strain numbers. The grey bar at the top of the figure shows the part (79.3% to 84.3%) of the ERIC fingerprints that is not taken into account to calculate the cluster, as explained in the text.

Genetic diversity (stability). Figure 3 shows the fingerprints of the 55 isolates belonging to 10 serotypes obtained with the ERIC primer set and the $(GTG)_5$ primer, as well as the clustering of the composite data set. With the ERIC primer set, 24 different clusters and/or separate strains were distinguished with 95% as the delineation level (data not shown). All strains belonging to serotype Agona or Indiana clustered together within the serotype. Serotype Blockley was divided into two clusters and/or separate strains and serotype Hadar was divided into three clusters and/or separate strains, although the profiles within one serotype were visually the same for both clusters. Serotypes Derby, Brandenburg and Virchow each had one strain (MB1736, MB1724 and MB2396, respectively) with an ERIC fingerprint that differed in one band from the other strains of the respective cluster. Serotype Infantis strains were divided into three clusters. The difference consisted of bands of more or less intensity at 400 bp. The nine strains of serotype Typhimurium clustered into two groups. The difference consisted of a double or single band at 700 bp. The serotype Enteritidis strains were divided into six clusters. Strain MB2499, strain MB1535 and strain MB1221 had each a different fingerprint from the other 10 strains. The other 10 strains were grouped together in three clusters, and the differences consisted in bands of different intensity at 2000 bp.

With the $(GTG)_5$ primer, 23 clusters and/or separate strains were distinguished when a delineation level of 94% was applied (data not shown). All strains belonging to the serotypes Hadar, Virchow and Indiana clustered together within the serotype. Each of the serotypes Infantis, Agona, Blockley and Brandenburg had one strain (MB1146, MB1230, KS163 and KS181, respectively) with a $(GTG)_5$ fingerprint profile that differed in one to three bands from the other strains of the cluster. Serotype Derby was divided into three clusters: two strains (MB1531 and MB1739) each had a fingerprint that differed in one high-intensity band from the other three strains. The nine serotype Typhimurium strains were divided into four clusters. The strains differed in the presence or absence of a band at 1200 or 600 bp. Strain MB2274 had a band of higher intensity than the other strains at 1600 bp. Eleven of the 13 strains of Enteritidis serotype were clustered together with a similarity coefficient of 94.6%, whereas the other two strains, MB1535 and MB2499, each had a different fingerprint.

Figure 3 shows the cluster of the composite data set of both primers and the fingerprints. With the delineation level of 92.5%, 21 different clusters and/or separate strains were distinguished. The strains belonging to serotypes Hadar, Indiana, Infantis and Virchow clustered together within the serotype. The strains of serotypes Brandenburg, Typhimurium and Agona were

divided into two clusters. Serotypes Blockley and Derby were split up into three clusters, whereas serotype Enteritidis was divided into five clusters.

4. DISCUSSION

To our knowledge this is the first study that has compared five different rep-PCR primers with regard to the potential of discriminating *Salmonella* serotypes. It is also the first study of the ability of primer $(GTG)_5$ to discriminate *Salmonella* serotypes. The reproducibility experiment indicated that the PCR run is more important for reproducibility than the lysates made at different times. This means that it is recommended to draw conclusions only from isolates that were processed in the same PCR run. An alternative is to include in the new PCR run some isolates (e.g., one from each cluster) that were processed in a previous PCR run.

All isolates generated fingerprints, including the five isolates which were not typeable by serotyping. All but a few serotypes had unique fingerprints. Strains MB2563 and MB2564, which were not typeable by serotyping, had identical fingerprints with both primers, meaning that they are probably genetically identical strains. Serotype Paratyphi B and one strain that was not typeable by serotyping also had identical fingerprints with both primers, although the similarity coefficient was below the delineation level for the ERIC primer set. Serotype Enteritidis, a strain that was not typeable by serotyping and a strain with antigenic formula 9:-:- had a similarity coefficient of 95% with the $(GTG)_5$ primer. This clearly shows that rep-PCR can reveal additional information when serotyping is not possible. Furthermore, it is possible that serotype Enteritidis and the strain with antigenic formula 9:-:- belongs to the same serotype but that some somatic and flagellar factors of the latter were not expressed during serotyping. The same can be concluded for serotype Infantis and MB2529, with antigenic formula 6,7:r:-, which had identical fingerprints with the ERIC primer set. Serotype Typhimurium ($O5^+$) and serotype Typhimurium var. Copenhagen ($O5^-$) produced the same fingerprint with the ERIC primer set. With the $(GTG)_5$ primer, a difference in one high-intensity band at 1200 bp was observed among the serotype Typhimurium strains tested, which, however, did not correlate with the two varieties in this serotype.

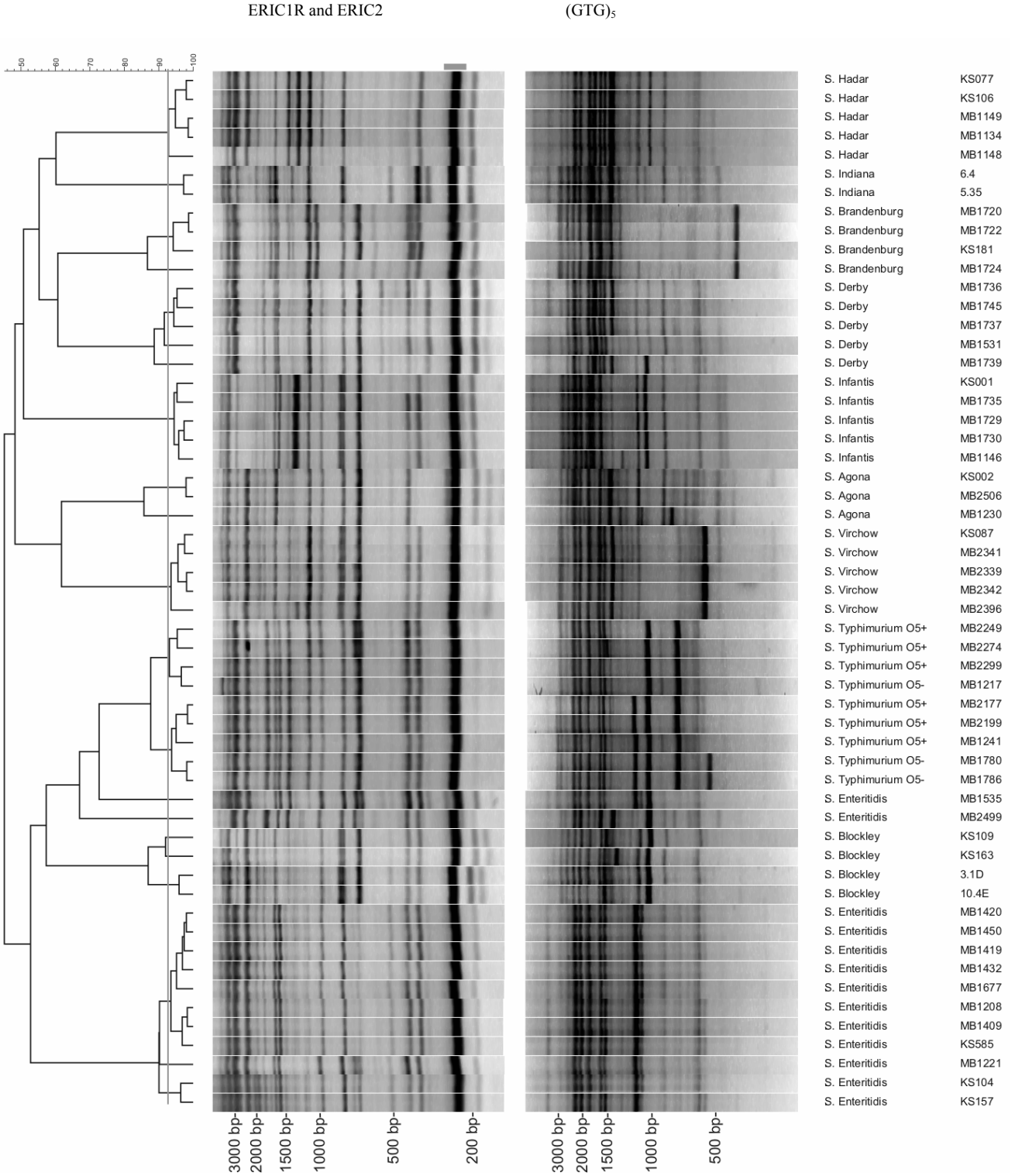


Figure 3. Cluster of the composite data set and ERIC and (GTG)5 fingerprints of 55 genetically different strains belonging to 10 serotypes. The similarities between the fingerprints were calculated using the Pearson correlation (optimization, 1%; position tolerance, 1%), and the fingerprints were grouped according to their similarities by use of the UPGMA algorithm. The vertical grey line shows the delineation level of 92.5%. The last column shows the strain numbers. The grey bar at the top of the figure shows the part (79.3% to 84.3%) of the ERIC fingerprints that is not taken into account to calculate the cluster, as explained in the text.

The genetic diversity (stability) experiment further showed that not all isolates with the same serotype had the same fingerprint. However, the isolates with the same serotype still clustered together at a similarity coefficient of 85% or higher, except for two serotype Enteritidis strains. Strain MB2499 was isolated from a reptile and strain MB1535 was isolated from a deer. Both were also atypical by other characterization methods such as randomly amplified polymorphic DNA and virulence typing (unpublished results). As mentioned by Torpdahl and Ahrens (2004), the serotype Enteritidis is a polyphyletic serotype. Although strains in this serotype are not genetically related, they share some characteristics like the somatic and flagellar factors.

The discriminatory powers of the ERIC primer set and the $(GTG)_5$ primer are similar, with 24 clusters (and/or separate strains) obtained by the former and 23 clusters (and/or separate strains) by the latter for a collection of 55 strains belonging to 10 serotypes. Nevertheless, this experiment revealed that the ERIC primer set and the $(GTG)_5$ primer are complementary since they did not discriminate the same strains within certain serotypes. This experiment clearly shows that one serotype does not always correspond to only one ERIC or $(GTG)_5$ fingerprint, but the fingerprint heterogeneity within a serotype seems to be limited to the absence or presence of mostly one and sometimes two bands for a primer or primer set or to differences in intensities of some bands. In a few restricted cases (e.g., MB1221 in ERIC-PCR of experiment 4; Figure 3), an apparent fingerprint heterogeneity seemed to be due to normalization artefacts. Nevertheless, this experiment indicates that direct serotype identification by rep-PCR may be erroneous if only one reference fingerprint is included.

Other studies have also evaluated the *Salmonella* discriminating ability of rep-PCR. Most studies (Burr *et al.,* 1998; Milleman *et al.*, 1996; Van Lith and Aarts, 1994) tested only the ERIC primer set, with conflicting results. According to Van Lith and Aarts (1994), it is possible to use the ERIC1R-ERIC2 primer set to discriminate *Salmonella* serotypes. Their study was performed on 65 *Salmonella* isolates of 49 serotypes. They concluded that all serotypes produced unique fingerprints and that the isolates within one serotype had identical patterns. According to Burr *et al.* (1998), who tested the same primer set on 89 *Salmonella* isolates of 22 serotypes, the fingerprints obtained did not correlate with serotypes. Milleman *et al.* (1996) also tested the ERIC primerset on 56 serotype Typhimurium and 14 serotype Enteritidis strains. They concluded that ERIC-PCR cannot be used to discriminate *Salmonella* serotypes, since all serotype Enteritidis isolates and some serotype Typhimurium isolates

shared the same fingerprint. According to two other studies (Johnson and Clabots, 2000; Johnson *et al.*, 2001), elevated annealing temperatures improve the reproducibility and resolving power of rep-PCR with ERIC2 and BOXA1R primers. In the first study only 12 strains of 12 serotypes and in the second study 70 isolates of 15 serotypes were evaluated. We obtained more bands with the ERIC primer set than were obtained in the studies mentioned above. This is probably the reason why some studies revealed that no serotype dependent fingerprints were obtained while other studies showed the opposite. The PCR conditions are probably critical factors, which also helps explain why in our study no serotype-dependent fingerprints were obtained at elevated annealing temperatures as described by Johnson and Clabots (2000).

It can be concluded that in certain epidemiological studies, ERIC-PCR and/or $(GTG)_5$ can be used to limit the number of strains that has to be serotyped, although it is useful only with isolates analyzed in one PCR run. Only one isolate of each cluster has to be sent to the national reference laboratories for serotyping. The reproducibility of isolates in one PCR run, the discriminatory power and the genetic diversity (stability) of the fingerprint are very similar for the ERIC primer set and the $(GTG)_5$ primer, so both primers are equally able to discriminate *Salmonella* serotypes. These techniques also produce fingerprints for nontypeable strains, which can be molecularly serotyped on the basis of the relationship to known serotypes. Moreover, they are also able to reveal serotyping errors.

CHAPTER II

The contribution of gastrointestinal colonization and cross-contamination to *Salmonella* carcass contamination during poultry slaughter

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Rasschaert G., K. Houf, C. Godard, C. Wildemauwe, M. Pastuszczak-Frąk, L. De Zutter. The contribution of gastrointestinal colonization and cross-contamination to *Salmonella* carcass contamination during poultry slaughter. *J. Food Prot.*: submitted

Summary

Successively slaughtered poultry flocks were sampled for *Salmonella* to study the relation between gastrointestinal colonization of the birds and contamination of the carcasses after slaughter and to examine cross-contamination. Samples from 56 broiler flocks and 16 spent laying hen and breeder flocks were collected in six slaughterhouses. *Salmonella* isolates were serotyped and further characterized by pulsed-field gel electrophoresis. Though only 7 (13%) broiler flocks were colonized with *Salmonella* at the time of slaughter, carcasses of 31 (55%) broiler flocks were contaminated after slaughter. Concerning the laying hen and breeder flocks, eleven flocks (69%) were colonized in the gastrointestinal tract, but after slaughter carcasses of all flocks were contaminated. Characterization of the isolates showed that the origin of the majority of the strains isolated from the carcasses was unknown since they did not originate from the gastrointestinal content of any of the flocks slaughtered that day. Cross-contamination was a major problem in different slaughterhouses. It was not only observed to following slaughtered flocks but also to preceding flocks. These observations make it clear that it is difficult to reach the aims of logistic slaughter in commercial poultry slaughterhouses.

1. INTRODUCTION

Salmonella is one of the most important foodborne pathogens in many countries. According to a report of the European Food Safety Authority (EFSA, 2006b), the mean number of human Salmonellosis cases in the 25 EU member states was 42 per 100 000 habitants in 2004, ranging from 7 per 100 000 habitants in Portugal to 300 per 100 000 habitants in the Czech Republic. In many industrialized countries, a steady increase in the number of human cases has been reported since the 1980s which is predominantly due to the increasing number of cases caused by the serotype Enteritidis (Rodrigue *et al.*, 1990). In 2004, *Salmonella* Enteritidis was the most common serotype in Europe with 76% of all human *Salmonella* isolates belonging to this serotype (EFSA, 2006b).

A wide range of food products are recognized as possible sources of human *Salmonella* infection, but poultry products have been identified as the most important (Rose *et al.*, 2000; Rose *et al.*, 2001 Kimura *et al.*, 2004). Vertical transmission of *Salmonella* from the laying hens and breeder flocks to eggs and chicks, respectively, has often been reported. This route is an important controlling factor in eradication programs via for example vaccination of the laying hen and breeder flocks and eradication of *Salmonella* infected parent flocks (Wegener *et al.*, 2003; EFSA, 2006a). However, horizontal transmission from the environment to poultry during the rearing period on the farm has also been reported as an important route (Heyndrickx *et al.*, 2002). Several risk factors for horizontal transmission have been identified such as a poor level of hygiene, the presence of rodents and insects on the farm, inadequate cleaning between rotation of flocks and contamination of the feed and drinking water (Davies and Wray, 1995; Davies and Wray, 1997; Rose *et al.*, 2000; Rose *et al.*, 2001; Davies and Breslin, 2003; Doyle and Erickson, 2006). At the time of slaughter, the gastrointestinal tract may harbor *Salmonella* and during the different stages of the slaughter process, the crop or intestinal content can be damaged and leach, causing a contamination on the carcasses. Furthermore, cross-contamination can occur from a *Salmonella*-positive flock or the slaughter equipment to the carcasses of a *Salmonella*-free flock (Carramiñana *et al.*, 1997; Lillard, 1990, Olsen *et al.*, 2003). A control measure to reduce this kind of cross-contamination is logistic slaughter which is applied in Belgium since 1999. The *Salmonella* status is determined by collecting samples of the fecal material in the poultry house by the veterinarian or the farmer a few weeks before slaughter. Flocks with a *Salmonella*-free status are

slaughtered first followed by *Salmonella*-positive flocks. However, Heyndrickx *et al.* (2002) demonstrated that carcasses of flocks with a *Salmonella*-free status were *Salmonella* contaminated after slaughter, even when they were processed first. These results indicate that logistic slaughter does not guarantee that the carcasses of *Salmonella*-free flocks are *Salmonella* free after slaughter.

The present study aimed first to determine the genotypic relation between *Salmonella* strains present in the alimentary tract and those on the carcasses after slaughter, and second, to study cross-contamination by sampling successively slaughtered poultry flocks.

2. MATERIALS AND METHODS

Slaughterhouses and flocks. The study was conducted in six Belgian poultry slaughterhouses from November 2001 to March 2004. Four slaughterhouses (A to D) processed only broiler chickens, whereas the two (E to F) slaughtered laying hen and breeder flocks. Each slaughterhouse was visited three times with a minimum interval of two weeks between the visits. All slaughterhouses were visited on Mondays after two days of no operation to minimize the risk of cross-contamination by the slaughter equipment. All slaughterhouses applied logistic slaughter and the status was determined two or three weeks before slaughter for the broiler flocks and between one week and one month before slaughter for the laying hen and breeder flocks.

In total, 72 flocks from 64 different farms were sampled. A flock was defined as all birds reared in the same poultry house on a farm. All farms applied an all-in, all-out system. The 56 broiler flocks were between 35 and 42 days old when slaughtered, whereas the 16 laying hen and breeder flocks were between 10 and 26 months old. Three flocks (E_2, E_3 and E_6) were breeder flocks, all other flocks were laying flocks. Eighteen flocks were foreign flocks; ten flocks were raised in the Netherlands (A_4, B_6, D_{10}, D_{11}, D_{12}, D_{13}, D_{16}, F_4, F_6 and F_{10}), four flocks were imported from France (B_2, B_7, B_{10}, E_4) and four flocks originated from Germany (F_2, F_3, F_5 and F_7). The remaining 54 flocks were raised in Belgium.

Sampling. Before slaughter, 30 living birds per flock were randomly selected from 15 crates from different transport containers. From each bird, a swab sample of the crop was taken. The swabs were pooled per 10 in a stomacher bag. Subsequently, from each flock 30

gastrointestinal tracts and 20 neck skin samples were collected immediately after evisceration and before chilling, respectively. For flocks with fewer than 4000 chickens, samples were randomly taken from the entire flock. For the other flocks, samples were taken randomly from approximately bird number 2000 to 8000. All samples were packed in sterile plastic bags and transported to the laboratory under cooled conditions and processed the same day.

Bacteriological culture. From each of the 30 gastrointestinal tracts, one g content of the duodenum and one g content of one cecum were aseptically collected. These samples were pooled, resulting in three subsamples of 10 g ceca content and three subsamples of 10 g duodenum content. The pooled samples were homogenized with 90 ml of buffered peptone water (BPW, Oxoid CM509, Basingstoke, UK) in a stomacher blender at normal speed. To each of the three bags with swabs, 25 ml BPW was added and homogenized manually. From each neck skin sample, 10 g was homogenized with 90 ml of BPW. All homogenates were incubated at 37°C for 16 to 20 h. From each pre-enrichment broth, 100 µl was plated onto diagnostic semi-solid *Salmonella* agar (Diassalm, LabM 537, Lancashire, UK) and 100 µl was added to 10 ml Rappaport-Vassiliadis bouillon (RV, Oxoid CM669, Basingstoke, UK). After incubation for 24 h at 42°C, the *Salmonella* suspected Diassalm plates and 10 µl of all RV tubes were plated onto xylose lysine deoxycholate (XLD, Oxoid CM469, Basingstoke, UK) and incubated at 37°C for 24 h. Presumptive *Salmonella* colonies were confirmed at genus level by PCR using the primers described by Aabo *et al.* (1993). The reaction mixture and amplification protocol were as described by Botteldoorn *et al.* (2003). One colony of each plate was stored in glycerol at -18°C for further examination.

Serotyping. All *Salmonella* isolates were characterized by enterobacterial repetitive intergenic consensus (ERIC) PCR. As described by Rasschaert *et al.* (2005), ERIC-PCR can be used to limit the number of strains that has to be serotyped as different strains belonging to the same serotype are clustered together with a delineation level of 95%. At least two isolates from each cluster were serotyped at the Belgian reference laboratory for *Salmonella* according to the Kauffmann-White scheme (Popoff and Le Minor, 1997).

Pulsed-field gel electrophoresis (PFGE). To characterize the *Salmonella* strains within serotypes, at least one isolate of each serotype isolated from each flock and from each sample type (neck skin, duodenum, cecum and crop) was further characterized by PFGE. These isolates were grown for 18 h on tryptone soya agar (TSA, Oxoid CM0131, Basingstoke, UK) at 37°C. The cells were suspended in cold Pett IV buffer (1M NaCl, 10mM Tris-HCl pH8,

10mM Na_2EDTA) and adjusted to an OD_{600} value of 0.8. The method of Olsen *et al.* (1994) was followed for preparing the plugs. Plug slices were digested for 18 h with 30 U of *Xba*I and *Not*I (Invitrogen, Paisley, UK) in single digestion reactions. DNA fragments were separated by Chefmapper in a 1% Seakem agarose (Biowhittaker Molecular Applications, Rockland, Maine, USA) gel. The running conditions were 6V/cm at 14°C in 0.5 x TBE buffer for 22 h with a ramping time from 4 to 40s for the *Xba*I enzyme or 24 h with a ramping time from 2 to 12 s for the *Not*I enzyme. PFGE profiles were clustered with GelCompar 3.0 (Applied Maths, Sint-Martens-Latem, Belgium) using the Dice coefficient (1% position tolerance) and the unweighted-pair group method using arithmetic averages algorithm (UPGMA). PFGE genotypes were assigned within serotypes on the basis of major polymorphisms, defined as a difference in the presence of at least one band in the *Xba*I and/or *Not*I fingerprint. The genotypes were indicated by numerical suffixes after a capital indicating the name of the serotype (e.g. H-1 refers to the serotype Hadar). Minor polymorphisms, defined as a small shift of one band was indicated by an apostrophe after the numerical suffix.

Phage typing. A selection of *Salmonella* Enteritidis isolates were phage typed by the National Phage Typing Centre of Belgium.

3. RESULTS

Broiler flocks. Of the 56 broiler flocks sampled, *Salmonella* was isolated from the crop or intestines from 7 flocks (13%) (A_5, A_6, A_8, B_7, C_2, D_{11} and D_{16}). Only three flocks (D_{11}, D_{16} and D_7) had received a *Salmonella*-positive status at the farm. From flock D_7, no *Salmonella* was isolated from the crop or intestinal tract at the time of slaughter (Table 1).

In total, 138 (12%) out of 1120 sampled neck skins were contaminated with *Salmonella* after slaughter. At flock level, 31 broiler flocks (55%) had 1 to 19 *Salmonella* contaminated neck skins (Table 1). Of the 128 neck skin isolates which were serotyped, 21.9% belonged to serotype Agona, 21.1% to serotype Hadar, 16.4% to serotype Infantis, 14.1% to serotype Typhimurium O5+, 8.6% to serotype Virchow, 6.3% to serotype Indiana, 3.9% to serotype Blockley, 3.1% to serotype Liverpool, 1.6% to serotype O4:d:- and 0.8% to the serotypes Ealing and Newport, respectively. Two isolates (1.6%) were not typeable (NT) by serotyping. Genotyping the neck skin isolates by PFGE resulted in 20 genotypes (Table 1).

From the seven flocks colonized with *Salmonella*, the neck skins of six flocks (A_5, A_6, B_7, C_2, D_{11} and D_{16}) were contaminated following slaughter (Table 1)**.** Only the slaughter of flock A_8, which was contaminated in the crop with strain NT-1, resulted in no contaminated neck skins. Flock A_5 as well as flock A_6 had only one contaminated neck skin, but the strain isolated from this neck skin did not correspond to the strain found in the intestines (Table 1). For the other four flocks, the *Salmonella* strains isolated from the neck skins did correspond to those isolated from the crop or the intestines. Flock B_7, which was slaughtered last that day, harbored serotype Hadar strain H-1 in the crop. Nineteen of the neck skins of this flock were contaminated with the same strain. Flock C_2, which was slaughtered as second flock that day, harbored serotype Typhimurium O5+ strain T-2 in the crop. This strain was isolated from some neck skins of this flock, but also from neck skins of the five flocks slaughtered subsequently. Both flock D_{11} as D_{16} harbored serotype Infantis in the gastrointestinal tract, but from another genotype. In both cases the flock was slaughtered last that day and the neck skins of these two flocks were contaminated with the same strain as isolated from the crop or intestines (Table 1). The neck skins of the flocks A_7, C_1 and D_{15} slaughtered just before flocks A_8, C_2 and D_{16}, respectively, were contaminated with the same *Salmonella* strains as isolated from the gastrointestinal tract from the following flock.

Laying hen and breeder flocks. Of the 16 laying hen and breeder flocks slaughtered in slaughterhouses E and F, *Salmonella* was isolated from the gastrointestinal tract of eleven (69%) flocks (Table 2). Two of these flocks (E_5 and F_8) had received a *Salmonella*-positive status at the farm level, whereas the status of eight of these flocks (E_4, F_2 to F_7 and F_{10}) was unknown since these flocks were foreign flocks from Germany, France or The Netherlands (Table 2). Flock E_4 was colonized with a strain which was not typeable by serotyping, whereas the ten other flocks were all colonized with serotype Enteritidis (Table 2). All flocks examined had contaminated neck skins after slaughter. Out of 320 sampled carcasses, 238 (74%) neck skins were contaminated with *Salmonella*. Of the 112 neck skin isolates which were serotyped, 82.8% belonged to serotype Enteritidis, 5.4% to serotype Agona, 5.4% to serotype Virchow, 3.6% to serotype Infantis, 1.8% to serotype Mbandaka and 1.8% to serotype Braenderup. Without taking into account small differences in profiles which are indicated by apostrophes as explained in Materials and Methods, ten PFGE genotypes were discriminated within serotype Enteritidis. The five other serotypes belonged to six different genotypes; only serotype Infantis could be divided in two genotypes (Table 2).

The neck skins of five flocks (F_2, F_3, F_6, F_7 and F_{10}) were contaminated with the same geno-/phage types as isolated from the gastrointestinal tract (Table 2). Cross-contamination from the crop or intestines of a flock to the neck skins of the following flock was observed only once. The neck skins from flock E_6 were contaminated with strain E-1 phage type 21 which was isolated from the crop and intestines from flock E_5.

Though not isolated from the crop or intestines from any of the flocks, the neck skins of some flocks slaughtered on the same day were contaminated with the same strain. Serotype Virchow strain V-3 was the only strain isolated from the neck skins of both flocks slaughtered on sampling day 14. The neck skins of flocks F_2 and F_4 were both contaminated with serotype Mbandaka strain M-1. Serotype Agona strain A-5 was isolated from the neck skins of flocks F_8, F_9 and F_{10}.

Table 1. Distribution of the *Salmonella* genotypes on the neck skins after slaughter and in the *Salmonella* colonized broiler flocks

		NECK SKINS			CROP			DUODENUM			CECA		
day	**flock**[a]	**number (/20)**	**serotype**	**PFGE**	**number**[b] **(/3)**	**serotype**	**PFGE**	**number**[b] **(/3)**	**serotype**	**PFGE**	**number**[b] **(/3)**	**serotype**	**PFGE**
1	A_1	3	Hadar	H-1[c]									
	A_2	2	Hadar	H-1									
	A_4	2	Hadar	H-1									
2	A_5	1	Indiana	I-1				3	Typhimurium O5+	T-1	1	Typhimurium O5+	T-1
	A_6	1	Newport	N-1				1	London	L-1			
	A_7	1	NT	NT-1									
	A_8							1	NT	NT-1			
3	A_{13}	2	ND										
5	B_6	1	Agona	A-1									
	B_7	19	Hadar	H-1	1	Hadar	H-1						
7	C_1	12	Agona (6)[d]	A-2									
			Virchow (2)	V-1									
			Typhimurium O5+ (1)	T-2									
	C_2	9	Agona (4)	A-2	1	Typhimurium O5+	T-2						
			Typhimurium O5+ (2)	T-2									
			Ealing (1)	Ea-1									
	C_3	8	Agona (2)	A-2									
			Typhimurium O5+ (2)	T-2									
			NT (1)	NT-2									
	C_4	6	Typhimurium O5+ (3)	T-2									
			Agona (1)	A-2									
			Agona (1)	A-3									
			O4:d:- (1)	O-1									
	C_5	5	Typhimurium O5+	T-2									
	C_6	3	Typhimurium O5+	T-2									
	C_7	3	Typhimurium O5+ (2)	T-2									
			O4:d:- (1)	O-1									

		NECK SKINS			CROP			DUODENUM			CECA		
day	**flock[a]**	**number (/20)**	**serotype**	**PFGE**	**number[b] (/3)**	**serotype**	**PFGE**	**number[b] (/3)**	**serotype**	**PFGE**	**number[b] (/3)**	**serotype**	**PFGE**
8	C_8	3	Virchow	V-1									
	C_9	4	Virchow (2)	V-1									
			Indiana (1)	I-2									
			Hadar (1)	H-1									
	C_{10}	4	Indiana (3)	I-2									
			Virchow (1)	V-1									
	C_{11}	3	Indiana	I-2									
	C_{12}	2	Virchow	V-1									
9	C_{15}	11	Agona	A-2									
	C_{16}	2	Agona	A-2									
10	D_5	2	Liverpool	Li-1									
	D_7	**2**	**Liverpool**	**Li-1**									
11	D_9	2	Agona	A-4									
			Infantis	Inf-1									
	D_{11}	**6**	**Infantis (5)**	**Inf-2**							**2**	**Infantis**	**Inf-2**
			Virchow (1)	**V-2**									
12	D_{12}	5	Blockley (3)	B-1									
			Infantis (2)	Inf-3									
	D_{14}	3	Blockley (2)	B-1									
			Infantis (1)	Inf-3									
	D_{15}	3	Infantis	Inf-1									
	D_{16}	**9**	**Infantis (5)**	**Inf-1**	**2**	**Infantis**	**Inf-1**						

[a]: only flocks colonized in the gastrointestinal tract and/or contaminated on the neck skins are included in the table. For the completeness: on day 2 flocks $A_5 \rightarrow A_9$ were slaughtered; on day 3 $A_{10} \rightarrow A_{13}$; on day 4 $B_1 \rightarrow B_4$; on day 5 $B_5 \rightarrow B_7$; on day 6 $B_8 \rightarrow B_{10}$; on day 9 $C_{13} \rightarrow C_{17}$; on day 10 $D_1 \rightarrow D_7$; on day 11 $D_8 \rightarrow D_{11}$; on day 12 $D_{12} \rightarrow D_{16}$. Flocks with a *Salmonella*-positive status at the farm are indicated in bold

[b]: pooled samples of 10 samples each

[c]: genotypes were indicated by numerical suffixes after a capital indicating the name of the serotype (e.g. H-1 refers to the serotype Hadar)

[d]: between brackets the number of neck skins of which the serotype was isolated. No number between brackets means that all neck skins were contaminated with the serotype.

NT: not typeable, ND: not determined

Table 2. Distribution of the *Salmonella* genotypes on the neck skins after slaughter and in the *Salmonella* colonized laying hen and breeder flocks

		NECK SKINS				CROP				DUODENUM				CECA			
day	**flock[a]**	**number (/20)**	**serotype**	**PFGE**	**phage type[b]**	**number[c] (/3)**	**serotype**	**PFGE**	**phage type**	**number[c] (/3)**	**serotype**	**PFGE**	**phage type**	**number[c] (/3)**	**serotype**	**PFGE**	**phage type**
13	E_1	4	Enteritidis	E-1' (2)[d, e]	4												
				E-2 (1)													
	E_2	6	Enteritidis	E-1 (2)	4												
				E-1' (1)													
14	E_3	2	Virchow	V-3													
	E_4	4	Virchow	V-3		1	NT	NT-3									
15	**E_5**	**20**	**Enteritidis (6)**	**E-1 (2)**	**35**	**2**	**Enteritidis**	**E-1**	**21**	**3**	**Enteritidis**	**E-1 (1)**	**21**	**1**	**Enteritidis**	**E-1**	1
					6a												
	E_6	19	Enteritidis (6)	E-1 (2)	21												
					6a												
				E-3' (1)	35												
16	F_1	20	Enteritidis (7)	E-3 (1)	21					3	Enteritidis	E-8	RDNC/P14b (1)				
				E-4 (1)									NT (2)				
				E-5 (1)													
			Infantis (3)	Inf-4 (1)													
	F_2	20	Enteritidis (9)	E-2 (1)		2	Enteritidis	E-1'	4	3	Enteritidis	E-1 (2)	21				
				E-1 (1)	21												
			Mbandaka (1)	M-1 (1)													
	F_3	20	Enteritidis (11)	E-6 (1)						3	Enteritidis	E-1 (1)	21	1	Enteritidis	E-9	8
				E-1 (2)	21												
	F_4	16	Enteritidis (10)	E-3' (1)						2	Enteritidis	E-1	21				
				E-7 (1)								E-1''	6a				
			Mbandaka (1)	M-1 (1)													

		NECK SKINS				CROP				DUODENUM				CECA			
day	**flock[a]**	**number (/20)**	**serotype**	**PFGE**	**phage type[b]**	**number[c] (/3)**	**serotype**	**PFGE**	**phage type**	**number[c] (/3)**	**serotype**	**PFGE**	**phage type**	**number[c] (/3)**	**serotype**	**PFGE**	**phage type**
17	F_5	17	Enteritidis (3)	E-1 (2)[d,e]	21					1	Enteritidis	E-10	4				
			Braenderup (2)	B-1													
	F_6	20	Enteritidis (11)	E-1' (1)	4					1	Enteritidis	E-1	4	1	Enteritidis	E-1'	4
	F_7	20	Enteritidis (5)	E-1' (2)	4	1	Enteritidis	E-1'	4	2	Enteritidis	E-1'	4	1	Enteritidis	E-2	2
			Infantis (1)	Inf-5 (1)													
18	**F_8**	**18**	**Enteritidis (8)**	**E-3 (1)**	**4**					**1**	**Enteritidis**	**E-1'**	**7a**				
				E-1' (1)	**4**												
			Agona (4)	**A-5 (1)**													
	F_9	16	Enteritidis (6)	E-1' (1)	4												
			Agona (2)	A-5 (1)													
	F_{10}	16	Enteritidis (9)	E-1' (1)	4	1	Enteritidis	E-3'		3	Enteritidis	E-1' (1)	4				
				E-4' (1)													
			Agona (1)	A-5 (1)													

[a]: flocks with a *Salmonella*-positive status at the farm are indicated in bold
[b]: phage typing: RDNC = routine dilution no conformity = unfound in the international reference table; 4=2/3/4/5/6/7/8/9/10/11/12/13; 6a=2/4/6/9; 7a=2/4/7/9/11/13 21=1/2/4/6/8/9/10/14; RDNC/P14b= 6/9/14
[c]: pooled samples of 10 samples each
[d]: genotypes were indicated by numerical suffixes after a capital indicating the name of the serotype (e.g. H-1 refers to the serotype Hadar)
[e]: between brackets the number of neck skins of which the serotype is isolated
ND: not determined
NT: not typeable

4. DISCUSSION

Of the 64 flocks of which the status was determined at the farm, five flocks had received a *Salmonella*-positive status. However, *Salmonella* was not recovered from one of these flocks at the time of slaughter. From six other flocks with a negative status, *Salmonella* was isolated from the gastrointestinal tract at the time of slaughter. This difference between the status at the farm and at the slaughterhouse can be the result of a change in the status in the weeks between status determination and processing the flock. The birds can change from *Salmonella* carriers to *Salmonella* shedders and *vice versa*, become apparently clear of infection or acquire a new infection (Olsen *et al.*, 2003). Rigby and Pettit (1980) have demonstrated experimentally that flocks transported in *Salmonella* contaminated crates became colonized. This contamination even spread to chickens placed in cleaned crates and transported in the same truck. Several studies have shown that between 13% and 99% of the transport containers are still contaminated with *Salmonella* after the cleaning and disinfection process (Rigby *et al.*, 1982; Olsen *et al.*, 2003) which makes it quite plausible that *Salmonella*-free flocks become infected during transport. Even more, colonization of the crop and/or duodenum as observed in some flocks could be due to a recent infection, since the ceca are the primary sites of colonization (Fanelli *et al.*, 1970).

Since the *Salmonella* status was sometimes incorrect, it was difficult to reach the benefits of logistic slaughter. For example, flock C_2 had received a *Salmonella*-negative status, but harbored at the time of slaughter a *Salmonella* strain in the crop. Neck skins of all following flocks were contaminated with this strain. Concerning the laying and breeding hen flocks, it seemed even more difficult to maintain logistic slaughter. A lot of foreign flocks were slaughtered, from which no status was determined. When the status was determined, the two slaughterhouses seemed not to take into account this status. Flocks E_5 and F_8 had both a *Salmonella*-positive status and were both slaughtered first before flocks with a negative or unknown status.

The slaughter of flocks colonized with *Salmonella* did not always result in the contamination of the neck skins. In slaughterhouse A, three *Salmonella*-positive flocks were slaughtered and the neck skins of none of these flocks were contaminated with the same strain as isolated from the intestines. The slaughter of the other four colonized flocks resulted in the contamination of the neck skins with the same strains as isolated from the gastrointestinal tracts. The slaughter

of flock B_7, which was only contaminated in the crop, even resulted in the contamination of nearly all neck skin samples. Whether or not the slaughter of *Salmonella*-positive flocks resulted in the contamination of neck skins, can have several causes. First, it can be slaughterhouse dependent. Only in slaughterhouse A, the slaughter of *Salmonella*-positive flocks did not result in the contamination of the neck skins. It is possible that in this slaughterhouse, more attention was paid to the adaptation of the slaughter equipment to the different sizes of the birds from different flocks leading to less pressure on the carcasses during plucking and less damaged intestines during evisceration. A second explanation is that the contamination is related to the place of the gastrointestinal tract where the birds are colonized. It is possible that crop colonization leads to more contaminated neck skins than colonization of the duodenum and ceca. Hargis *et al.* (1995) have demonstrated that during processing, the crop is more than 80 times more likely to rupture than the ceca. A last explanation is that the contamination is strain dependent: some strains are better adapted to surviving environmental stress in the slaughterhouse and to contaminate neck skins and the slaughter line (Olsen *et al.*, 2003).

On some occasions, cross-contamination was observed. Flock C_2 for example, was colonized in the crop with a certain *Salmonella* strain. The neck skins from this flock but also the neck skins from the five subsequently slaughtered flocks were – among other strains- contaminated with this strain. The present study shows not only cross-contamination to following flocks but also in a few cases to preceding flocks. Allen *et al.* (2003) demonstrated forward contamination due to aerosol contamination in the plucking machine. A seeder carcass inoculated with an *E. coli* strain did not only contaminate following carcasses but also all sampled preceding carcasses. However, only 30 preceding carcasses were sampled and as a flock consists of several thousands birds, it should be thoroughly investigated how many carcasses preceding a *Salmonella* colonized flock can become contaminated. Even when only *Salmonella*-free flocks were slaughtered or when logistic slaughter was applied correctly, carcasses of several flocks were contaminated with certain S*almonella* strains of which the origin was not clear. This external contamination may have originated from the transport containers or the slaughter environment as demonstrated by Rigby *et al.* (1980), Corry *et al.* (2002) and Olsen *et al.* (2003).

From all neck skin isolates serotyped, nearly 40% belonged to serotype Enteritidis. These isolates originated all from laying hen flocks. Ten out of 13 (77%) laying hen flocks were

colonized with *Salmonella* Enteritidis. This *Salmonella* Enteritidis prevalence is much higher than the prevalence reported in an EFSA study (EFSA, 2006a) in which the European mean of *Salmonella* colonized flocks 30% is. However, in the study of EFSA, feces and environmental samples were collected at the farm during the last 9 weeks of production of the flocks. As explained above, the status can change in the last weeks, days or even hours before processing. Furthermore, Belgian farmers are strongly encouraged to vaccinate laying hen and breeder flocks against *Salmonella* Enteritidis. Within time, this study should be repeated to evaluate the impact of this vaccination on the contamination level of carcasses of laying hen and breeder flocks following slaughter.

In conclusion, the present study has revealed several problems regarding logistic slaughter in Belgian commercial slaughterhouses. First, when the status of a flock was incorrect or unknown, it was difficult to reach the expected benefits of logistic slaughter. Second, when only *Salmonella*-free flocks were slaughtered or slaughtered first, neck skins of the flocks were still contaminated with strains from which the origin was not clear. They originated probably from the slaughter environment or the transport crates. Third, not only cross-contamination to the following flocks was observed but also to preceding flocks.

CHAPTER III

The contribution of gastrointestinal colonization and cross-contamination to *Campylobacter* carcass contamination during poultry slaughter

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Adapted from

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Summary

The relation between internal carriage and surface contamination with thermophilic *Campylobacter* species in broilers was examined by molecular typing methods. Samples from 39 flocks were collected in three Belgian poultry slaughterhouses. From each flock, crop swabs before slaughter and intestines and neck skins during slaughter were collected. A total of 309 isolates were identified at species level and further characterized by flagellin gene A PCR/restriction fragment length polymorphism and pulsed-field gel electrophoresis. Isolates were identified as *Campylobacter jejuni* (89%), *Campylobacter coli* (8.7%) and *Campylobacter lari* (2.3%), and 27 genotypes could be distinguished by combining the two molecular methods. Seventy-two percent of the flocks arriving at the abattoir were colonized with campylobacters. After slaughter, 79 % of the flocks had contaminated neck skins. In six flocks, genotypes isolated from the neck skins were also found in the alimentary tract from previously slaughtered flocks. Four of these flocks were initially free of *Campylobacter*. These four flocks might have had no contaminated carcasses after logistic slaughtering.

1. INTRODUCTION

The thermophilic *Campylobacter* species are leading causative agents of bacterial enteritis in the developed countries (Tauxe, 1992). Consumption or mishandling of raw or undercooked poultry products have been identified as major risks for infection (Pearson *et al.*, 2000; Vellinga and Van Loock, 2002). The initial source of colonization of broilers is still unidentified. According to most studies, the birds become colonized by horizontal transmission during rearing. The lack of a hygiene barrier, dirty footwear, rodents and insects, thinning of the flock, inappropriate cleaning of the broiler house or a too-short down period between flocks have been identified as risk factors for transmission (Berndtson *et al.*, 1996a; Hald *et al.*, 2001; Petersen and Wedderkopp, 2001; Herman *et al.*, 2003). Once introduced, campylobacters spread very quickly throughout the broiler house, perhaps via drinking nipples (Berndtson *et al.,* 1996a) and coprophagic behavior (Newell and Fearnley, 2003). At the age of five to six weeks, the birds are loaded into crates and transported to the slaughterhouse. These crates are washed and disinfected after use. However, inadequately washed and disinfected crates have been identified as an additional source of contamination for chickens subsequently transported in such crates. As shown by the study of Newell *et al.* (2001), carcasses from *Campylobacter*-free chickens can be contaminated with strains present in the crates used to transport the birds to the slaughterhouse.

During slaughter, carcass contamination can occur during the plucking and the evisceration process. The rubber fingers applied in the defeathering process exert pressure on the carcasses, forcing potential contaminated fecal material out and spreading it on the carcasses and the slaughter equipment (Oosterom *et al.*, 1983; Berrang *et al.,* 2001). During evisceration, the intestines can rupture and leak fecal material (Izat *et al.*, 1988). As a consequence, the surfaces of carcasses from a *Campylobacter*-free flock can be contaminated by previously slaughtered flocks during slaughter.

The aim of the study was to determine the relation between campylobacters present in the alimentary tract and on the poultry carcasses by molecular characterization to examine potential contamination sources for successive flocks.

2. MATERIALS AND METHODS

Sampling. The study was conducted in three unrelated Belgian poultry slaughterhouses (A, B and C[1]) from January to July 2002. Each slaughterhouse was visited three times, with a minimum interval of two weeks between visits. The slaughterhouses were visited on Mondays after two days of no operation. Only flocks slaughtered in the morning were sampled. Plants A, B and C had processing capacities of 9000, 6000 and 6000 birds per hour, respectively. The broilers were scalded at 51°C in all plants. After defeathering, the carcasses entered the evisceration line, where the intestines were removed mechanically by clamps or spoons. The carcasses were cleaned by an inside-outside washer just before air chilling.

In total, 39 broiler flocks from 37 different farms were examined. A flock was defined as all birds reared in the same poultry house on a farm. All farms applied an all-in, all-out system. Birds were between 35 and 42 days old when slaughtered. Flock size ranged from between 1100 and 18000 chickens. In slaughterhouse A, B and C, 13 (A_1 to A_{13}), 10 (B_1 to B_{10}), and 16 (C_1 to C_{16}) flocks were sampled. Flocks A_4 and B_6 were raised in The Netherlands; flocks B_2, B_7 and B_{10} were raised in France. The remaining 34 flocks were raised on Belgian farms, located throughout the country. Flocks A_1 and A_2 and flock A_{11} and A_{12} were reared at the same time in different poultry houses on the same farm.

From each flock, 30 living birds were randomly selected from 15 crates from different transport containers. Of each bird, one swab sample of the crop was taken and stored and transported in buffered peptone water (BPW, Oxoid CM509, Basingstoke, UK). Additionally, from each flock, 30 gastrointestinal tracts and 20 neck skin samples were randomly collected immediately after evisceration and before chilling, respectively. For flocks with fewer than 4000 chickens, samples were randomly taken from the entire flock. For the other flocks, samples were taken randomly from bird number 2000 to 8000. All samples were packed in sterile plastic bags, transported to the laboratory under cooled conditions and processed the same day.

[1] Slaughterhouses A, B and C are the same slaughterhouses as A, B and C in Chapter II. Moreover, the same flocks were sampled.

Bacteriological culture. From each of the 30 gastrointestinal tracts, 1 g content of the duodenum and 1 g content of one cecum were aseptically collected. These samples were pooled to create three subsamples of 10 g duodenum content and three subsamples of 10 g cecum content. The pooled samples were homogenized with 90 ml of BPW in a stomacher blender at normal speed. Thirty crop swabs were pooled to three subsamples of 10 swabs each and homogenized with 25 ml of BPW. From each neck skin sample, 10 g was homogenized with 90 ml of BPW. Of each of the homogenates, 1 ml was added to 9 ml selective Preston broth (nutrient broth n°2, Oxoid CM67, Basingstoke, UK, enriched with 5% (vol/vol) lysed defibrinated horse blood and 1% Preston supplement (5000 IU polymixin B, 0.010 g rifampicin, 0.0076 g trimethroprim and 0.010 g amphotericin dissolved in 10 ml of ethanol)). The homogenates were incubated for 24 to 48 h at 42°C under microaerobic conditions by evacuating 80% of the normal atmosphere and introducing a gas mixture consisting of 8% CO_2, 8% H_2 and 84% N_2 into each jar. After 24 h, 10 µl of each enrichment broth was plated onto modified cefoperazone charcoal deoxycholate agar (mCCDA, CM739 plus SR155, Oxoid, Basingstoke, UK) and incubated for 24 to 48 h at 42°C under microaerobic conditions. If there was no *Campylobacter* growth on mCCDA after 24 h of incubation, 10 µl of the 48-h incubated enrichment broth was plated on a new mCCDA plate. Morphologically typical colonies were picked, examined by Gram-staining and subcultured on a blood agar plate (Oxoid CM965 and L13, Basingstoke, UK and 5% (vol/vol) defibrinated horse blood). One colony of each of the pooled samples of the crops, the small intestines and the ceca and a maximum of nine isolates of the neck skin samples were stored in whole horse blood at –80°C for further examination.

Species identification and characterization of *Campylobacter* isolates. All isolates were identified at species level according to the PCR assay of Vandamme *et al.* (1997) and the PCR assay of Linton *et al.* (1996). All isolates were further characterized by flagellin gene A PCR/restriction fragment length polymorphism (*fla*A typing). The method of Nachamkin *et al.* (1993) with the use of *Dde*I (Promega, Madison, Wisconsin, USA) as the restriction enzyme was applied.

For each *fla*A type within one flock, at least one isolate from the crops, one from the small intestines, one from the ceca and one from the neck skins were randomly chosen for further characterization by pulsed-field gel electrophoresis (PFGE) with *Sma*I as the restriction enzyme (Invitrogen, Paisley, Scotland, UK). These isolates were grown for 18 h on mCCDA

at 42°C under microaerobic conditions. The cells were suspended in cold Pett IV buffer (1M NaCl; 10mM Tris-HCl pH8; 10mM Na_2EDTA) and adjusted to an optical density at 600 nm (OD_{600}) value of 0.55. For preparing the plugs, the method of Olsen *et al.* (1994) was followed. Plug slices were digested for 18 h with 40 U of *Sma*I. DNA fragments were separated by Chefmapper in a 1% Seakem agarose (Biowhittaker Molecular Applications, Rockland, Maine, USA) gel. The running conditions were 6 V/cm at 14°C in 0.5 x Tris-Borate-EDTA buffer for 22 h with a ramping time from 4 to 40 s. A second PFGE analysis with *Kpn*I was performed on isolates for which no fingerprint was generated by *fla*A or PFGE (*Sma*I) and on isolates with the same fingerprint type (*fla*A or PFGE (*Sma*I)) but from different flock origin. DNA was digested for 18 h with 40 U of *Kpn*I and run for 19 h with a ramping time from 4 to 20 s. All gel images were analyzed with GelCompar version 3.0 (Applied Maths, Sint-Martens-Latem, Belgium). The similarities between the fingerprints were calculated for both *fla*A and PFGE analysis using the band-based Dice coefficient, with an optimization and position tolerance of 1%. The fingerprints were grouped according to their similarities by use of the UPGMA (unweighted-pair group method using arithmetic averages) algorithm.

Delineation of a genotype. A delineation level of 90% was used to discriminate different genotypes. Isolates from the same flock with fingerprints obtained by one characterization method that differed in one to three fragments were considered closely related if no differences in the fingerprint with the other typing method were observed. They are indicated with an asterisk (Figure 1).

Statistical analysis. The number of contaminated neck skins was compared between the three slaughterhouses by the chi-square test.

3. RESULTS

Prevalence data obtained on the different sampling days in slaughterhouses A, B and C are shown in Table 1. From 11 (28%) flocks, no campylobacters were isolated from the crops, the duodena, or the ceca. These flocks were considered *Campylobacter* free. Three of these *Campylobacter*-free flocks were slaughtered in slaughterhouse A on the second sampling day, and eight flocks were slaughtered in slaughterhouse C distributed over the three days of sampling. No *Campylobacter*-free flocks were slaughtered in slaughterhouse B.

After slaughter, no campylobacters were found on the neck skins of eight flocks (21%). Seven of these eight flocks belonged to the 11 flocks that were considered as *Campylobacter* free. The remaining four flocks that were initially *Campylobacter* free (10%) had 1 to 18 contaminated neck skins (Table 1).

On average, 73% of the neck skins in slaughterhouse A, 87% in slaughterhouse B and 34% in slaughterhouse C were contaminated with campylobacters. The data set with the number of contaminated neck skins was compared between the three slaughterhouses, but the 11 flocks that were considered *Campylobacter* free were not taken into account for the calculations. Contamination of the neck skins in slaughterhouse C was significantly different ($p<0.05$) from contamination of the neck skins in slaughterhouses A and B.

In total, 309 isolates were identified at species level. Two hundred seventy-five (89%) isolates were identified as *C. jejuni*, 27 (8.7%) as *C. coli* and 7 (2.3%) as *C. lari*. In abattoir A, three successively slaughtered flocks harbored *C. lari* in the small intestines, ceca, or both. *C. coli* was isolated from the small intestines, ceca, or both in one flock slaughtered in abattoir A and in three flocks slaughtered in abattoir C. All isolates of flocks slaughtered in abattoir B were identified as *C. jejuni*. Flocks, contaminated only with *C. jejuni*, had a high contamination rate of neck skins (average 17 of 20), whereas the two flocks C_7 and C_{13} colonized with *C. coli* had 3 of 20 and 4 of 20 contaminated neck skins, respectively. In a mixed contamination of *C. jejuni* and *C. coli*, as in flock A_7 and C_4, all sampled neck skins were contaminated and isolates were identified as *C. jejuni*.

Characterizing the 309 isolates with *fla*A genotyping resulted in 22 different *fla*A genotypes (indicated by arabic numbers, Table 1). A total of 133 isolates were further characterized with

PFGE with the use of *Sma*I restriction enzyme (indicated by letters, Table 1). Twenty-five genotypes were distinguished by PFGE. Combining the two methods resulted in 27 genotypes. PFGE genotype r, identified as *C. lari*, was not typeable by *fla*A genotyping. Strains with *fla*A genotype 15 generated a fingerprint by PFGE with *Kpn*I, but not with *Sma*I.

In general, the genotypes identified were unique within one slaughterhouse. Four genotypes were found in flocks slaughtered in different abattoirs: genotype 6.c was found in the crop of flocks A_{10} and A_{11} and in the crops, the duodena and the ceca of flock B_8. Flock B_9 carried genotype 21.d in the intestines which was also found in the intestines of flock C_5. Flock B_3 and flock C_4 harbored genotype 15.- in their intestines. One isolate of the neck skins from flock C_{14} was the same genotype 2.b that was isolated from the crops and the duodena of flock A_7. Subsequent characterization by PFGE with restriction enzyme *Kpn*I confirmed the genotypes already delineated.

Genotypes isolated from the neck skin samples were also found in the crops, the duodena, or the ceca from the same flock or from one or two previously slaughtered flocks. In flock A_{10}, the origin of the contamination of the neck skins could not be determined: all isolates from the neck skins were genotype 7.c, which was different from those isolated from the intestines (5.d) or the crops (6.c). This flock was the first flock slaughtered that day. In flock $C_{14,}$ two genotypes, 2.b and 26.n, which were not isolated from the crop or the intestines, were isolated from the neck skins (Table 1).

In two cases, two flocks were raised on the same farm: flocks A_1 and A_2 and flocks A_{11} and A_{12}. Flocks A_1 and A_2 both harbored genotype –.r, identified as *C. lari*, in their intestines. The same genotype was isolated from the duodena and ceca of flock A_3, which was raised on another farm. Flock A_{11} carried genotype 6.c in the crops, which was also found in the crops of flock A_{10}. Flock A_{12} harbored genotype 7.e in the crops and the intestines, which was also isolated from flock A_{13}. The same observation -flocks from different origin but slaughtered on the same day, harboring the same genotype in their intestines- was made on three other sampling days. Flocks C_8 and C_9 shared subtype 24.t in their intestines, which was also found in the crops and the duodena of flock C_{14}, which was slaughtered in the same establishment but two weeks later. From the crops or the duodena of flocks B_1, B_3 and B_4, genotype 13.b could be isolated. Flocks B_5, B_6 and B_7 shared genotype 28.u in the crops, duodena, or ceca. In each case, this was also confirmed by PFGE with the restriction enzyme *Kpn*I.

Table 1. Prevalence of *Campylobacter* obtained on the nine different sampling days in slaughterhouse A, B and C

	CROP			DUODENUM			CECUM			NECK SKIN		
	number [a]	**species**[b]	**genotype**[c]	**number**	**species**	**genotype**	**number**	**species**	**genotype**	**number**	**species**	**genotype**
Abattoir A												
day 1												
A_1	0/3			1/3	*C. lari*	-.r[d]	3/3	ND	ND	4/20	ND	ND
A_2	1/3	*C. jejuni*	6.j	3/3	*C. lari*	-.r	3/3	ND	ND	20/20	*C. jejuni* (6)	6.j
A_3	2/3	ND	ND	1/3	*C. lari*	-.r	3/3	C. *jejuni* (1) C. lari (2)	6.j -.r	19/20	*C. jejuni* (5)	6.j
A_4	0/3			1/3	ND	ND[e]	3/3	ND	ND	9/20	*C. jejuni* (6)	6.j
day 2												
A_5	1/3	*C. jejuni*	1.f	3/3	*C. jejuni*	1.f	3/3	C. *jejuni*	1.f	18/20	*C. jejuni* (7)	1.f
A_6	0/3			0/3			0/3			0/20		
A_7	3/3	*C. jejuni*	2. b*[f]	3/3	C. *jejuni* (2) C. *coli* (1)	2.b 3.a	3/3	C. *coli*	3.a	20/20	*C. jejuni* (5)	2.b
A_8	0/3			0/3			0/3			18/20	*C. jejuni* (7)	2.b*
A_9	0/3			0/3			0/3			7/20	*C. jejuni* (6) *C. coli* (1)	2.b 3.a
day 3												
A_{10}	2/3	*C. jejuni*	6.c	3/3	*C. jejuni*	5.d	1/3	*C. jejuni*	5.d	16/20	*C. jejuni* (7)	7*.c
A_{11}	3/3	*C. jejuni*	6.c	3/3	*C. jejuni*	8.g	3/3	*C. jejuni*	8.g	20/20	*C. jejuni* (6)	6.c (5) 8.g (1)
A_{12}	3/3	*C. jejuni*	7.e	3/3	*C. jejuni*	7.e	3/3	*C. jejuni*	7.e	20/20	C. *jejuni* (6)	7.e (5) 6.c (1)
A_{13}	3/3	*C. jejuni*	7.e	3/3	*C. jejuni*	7.e	2/3	*C. jejuni*	7*.e	20/20	*C. jejuni* (6)	7.e
Abattoir B												
day 4												
B_1	1/3	*C. jejuni*	12. l*	3/3	*C. jejuni*	12.l (2) 13.o (1)	0/3			5/20	*C. jejuni*	12.l (4) 13.o* (1)
B_2	2/3	*C. jejuni*	1.h	3/3	*C. jejuni*	1.h	0/3			20/20	*C. jejuni* (6)	1.h
B_3	3/3	*C. jejuni*	13.o	3/3	*C. jejuni*	13.o (2) 15.- (1)	0/3			20/20	*C. jejuni* (5)	13.o (2) 15.- (3)
B_4	0/3			1/3	*C. jejuni*	13.o	0/3			20/20	*C. jejuni* (6)	13.o
day 5												
B_5	2/3	*C. jejuni*	28.u	3/3	*C. jejuni*	28.u	1/3	*C. jejuni*	28.u	20/20	*C. jejuni* (6)	28.u (3) 29.x (3)
B_6	ND			3/3	*C. jejuni*	29.x	3/3	*C. jejuni*	28.u 29.x	20/20	*C. jejuni* (6)	29.x
B_7	0/3			1/3	*C. jejuni*	28.u	1/3	*C. jejuni*	28.u	9/20	*C. jejuni*	28.u (7)

	CROP			DUODENUM			CECUM			NECK SKIN		
	number[a]	**species**[b]	**genotype**[c]	**number**	**species**	**genotype**	**number**	**species**	**genotype**	**number**	**species**	**genotype**
												29.x (2)
day 6												
B_8	2/3	*C. jejuni*	6.c	3/3	*C. jejuni*	6.c	3/3	*C. jejuni*	6.c	20/20	*C. jejuni* (6)	6.c
B_9	0/3			3/3	*C. jejuni*	21.d	2/3	*C. jejuni*	21.d	20/20	*C. jejuni* (6)	21.d
B_{10}	2/3	*C. jejuni*	36.z	3/3	*C. jejuni*	36.z	2/3	*C. jejuni*	37.k	20/20	*C. jejuni* (2)	36.z
Abattoir C												
day 7												
C_1	1/3	ND	ND	3/3	ND	ND	1/3	ND	ND	19/20	ND	ND
C_2	0/3			0/3			0/3			0/20		
C_3	0/3			0/3			0/3			0/20		
C_4	3/3	*C.jejuni*	17.q (2) 7.m (1)	3/3	*C. jejuni*	17.q 15.- 7.m	3/3	*C. jejuni* (2) *C. coli* (1)	17. q* 15.- 19.x	20/20	*C. jejuni* (6)	7.m
C_5	3/3	*C. jejuni*	20.v	3/3	*C. jejuni*	21.d	3/3	*C. jejuni*	20.v 21.d	20/20	*C. jejuni* (6)	20.v (5) 21.d (1)
C_6	0/3			0/3			0/3			11/20	*C. jejuni* (4)	7.m (1) 20*.v (3)
day 8												
C_7	0/3			2/3	*C. coli*	3.s	3/3	*C. coli*	3.s	3/20	*C. coli*	3.s
C_8	0/3			2/3	*C. jejuni*	24.t	2/3	*C. jejuni*	24.t	11/20	*C. jejuni* (6)	24.t
C_9	0/3			1/3	*C. jejuni*	24.t	0/3			0/20		
C_{10}	0/3			0/3			0/3			0/20		
C_{11}	0/3			0/3			0/3			0/20		
day 9												
C_{12}	0/3			0/3			0/3			0/20		
C_{13}	0/3			3/3	*C. coli*	25.w	3/3	*C. coli*	25.w	4/20	*C. coli*	25.w
C_{14}	2/3	*C. jejuni*	24.t	3/3	*C. jejuni*	24.t (2) 35.y (1)	3/3	*C. jejuni*	35.y	19/20	*C. jejuni* (7)	24.t (3) 26.n (2) 2.b (1) 35.y (1)
C_{15}	0/3			0/3			0/3			1/20	*C. jejuni*	24.t
C_{16}	0/3			0/3			0/3			0/20		

[a] = number of positive cultures/total number of samples; [b] = the number of isolates identified at the species level is parantheses; no number indicates that all isolates were identified at the species level; [c] = *fla*A type with *Dde*I as the restriction enzyme indicated by a number, and PFGE with *Sma*I as the restriction enzyme indicated by a letter; [d] = -, Not typeable, [e] = ND, Not Done; [f] = the asterisk indicates that one of the *fla*A or PFGE fingerprints is slightly different (see text) from the others.

Of the 133 isolates that were typed both by *fla*A and PFGE (*Sma*I), at least one isolate had a slightly different *fla*A or PFGE fingerprint for 8 genotypes (Table 1). For each of the genotypes 7.e, 7.c and 20.v, there was one isolate that had a *fla*A fingerprint very similar to the genotype, but had the same PFGE fingerprint (*Sma*I and *Kpn*I). The same was observed for the PFGE fingerprints for genotypes 2.b, 12.l, 13.o and 17.q. For genotype 2.b, two isolates each had a different PFGE fingerprint. One of these fingerprints was the same as genotype 13.o; the other fingerprint was the same as fingerprint 13.o* (Figure 1 and Table 1).

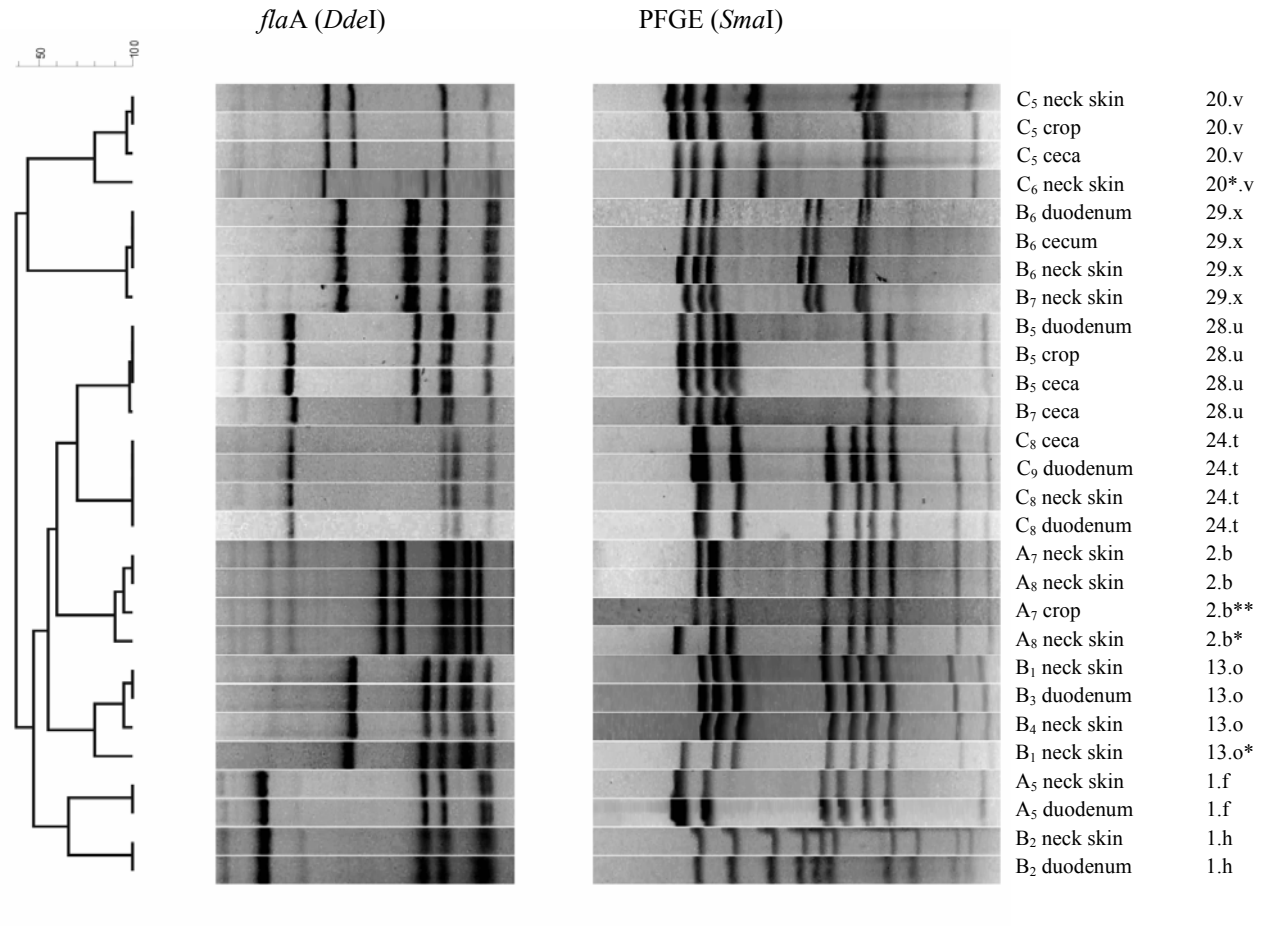

Figure 1. Example of a cluster of the composite data set and the *fla*A and PFGE fingerprints. A delineation level of 90 % was applied to discriminate the different genotypes. Closely related isolates are indicated by asterisks. The text columns show the flock number, the isolation site and the genotype of the isolate.

4. DISCUSSION

In this study, 72% (n=39) of the flocks arriving at the abattoir were colonized with campylobacters. This prevalence is similar to another Belgian study with 67% of the flocks (n=18) slaughtered carrying campylobacters in the ceca (Herman *et al.*, 2003) and a Dutch study with a prevalence of 82% (n=187) (Jacobs-Reitsma *et al.*, 1994). Other studies reported prevalence of flocks colonized with campylobacters at farm level. This prevalence ranges from 27% (n=287) in Sweden (Berndtson *et* al., 1996) to more than 90% (n=100) in the United Kingdom (Evans and Sayers, 1997). This difference could be due to different isolation methods, health herd management on the farms and probably the season in which the chickens were raised. Several studies mention a seasonal variation in the prevalence of poultry flock colonization (Jacobs-Reitsma *et al.*, 1994; Berndtson *et al.*, 1996b). This seasonal variation is expressed by a higher rate of colonization in summer than in winter. In this study, the flocks considered *Campylobacter* free were all raised from December until April, whereas the flocks colonized with campylobacters were reared from December until July.

To our knowledge this is the first extended study in which the sources of cross-contamination in a poultry slaughterhouse were studied by *fla*A and PFGE. Several successively slaughtered flocks were sampled and examined for the presence of campylobacters in 0.1 g of neck skin or intestinal content (detection level of 10 CFU/g) and isolates were characterized at strain level. The contamination on the carcasses correlated with the carriage of campylobacters in the crops, the duodena, or the ceca of the chickens just before slaughter. However, it seems that *Campylobacter* genotypes isolated from the neck skins are more often found in the crops or the duodena than in the ceca, which might indicate that the crop or the duodena are more important sources of carcass contamination during slaughter. During slaughtering, ceca are rarely damaged, whereas the small intestines are frequently ruptured.

Carcass contamination by the intestinal content of birds within a flock during transport and slaughter seems to be more prevalent than cross-contamination by previously slaughtered flocks. Only two flocks (A_{12} and B_7), which were carrying campylobacters in their intestines, had contaminated neck skins with genotypes also isolated from the crops or the intestines of the previously slaughtered flocks. Four flocks (A_8, A_9, C_6 and C_{15}), which were considered *Campylobacter* free also had neck skins contaminated with genotypes isolated from previously slaughtered flocks. These four flocks might have had no contaminated carcasses

after logistic slaughtering. Evidence of cross-contamination was also provided by genotyping the isolates from two (Newell *et al.*, 2001) and three (Rivoal *et al.*, 1999) subsequently slaughtered flocks at the abattoir. During slaughter, equipment becomes contaminated with campylobacters originally present in the crop or intestines. Some genotypes, such as 2.b, can be carried over onto the carcasses of subsequent flocks, whereas other genotypes such as 1.f, seem not be able to survive the same environmental stresses, as observed in other studies (Newell *et al.*, 2001). Two flocks (C_7 and C_{13}), contaminated with *C. coli* had fewer contaminated neck skins compared with other flocks that were contaminated with C. *jejuni*. One of the possible reasons is that *C. jejuni* is more able to survive the environmental stresses in the slaughterhouse than *C. coli*. However, this requires further investigation.

Only four genotypes (2.b, 6.c, 21.d, 15.-) were found in more than one slaughterhouse, which could indicate that some genotypes are predominant in Belgium. However, this cannot explain why flocks, slaughtered on the same day in the same slaughterhouse, harbored the same genotype in crops or intestines. Because the flocks are reared on different farms, the possibility that these flocks carry the same genotype in their intestines is small unless the different farms were in contact when the chickens were being raised. Flocks B_1, B_3 and B_4 all harbored the same genotype in their intestines. The farms on which these flocks were raised were all supplied by the same feed supplier and were all visited by the same veterinarian within two weeks before slaughter. Flocks A_1 and A_2, which were raised in different houses on the same farm, and flock A_3 harbored the same genotype of *C. lari* in their intestines, but flock A_3 was not in contact with the other two flocks during raising. Flocks A_{10} and A_{11} and flocks A_{12} and A_{13} also carried the same genotype in their intestines. Nevertheless, only flocks A_{10}, A_{11} and A_{12} were in contact via the feed supplier. Flock B_5, B_6 and B_7 were raised in farms located in three different countries. The possibility that these flocks were in contact during rearing was small.

The three slaughterhouses have their own lorries and transport containers. Newell *et al.* (2001) and Slader *et al.* (2002) showed that during transport birds can become surface contaminated with campylobacters excreted by the flock transported previously but still present in the washed and disinfected crates. These campylobacters are found on the processed carcasses. Herman *et al.* (2003) showed that 5 of 11 flocks, from which no campylobacters were isolated from cecal droppings at the farm, harbored campylobacters in the ceca during slaughter. According to Jacobs-Reitsma and Bolder (1998), it is possible that

improperly cleaned crates contaminate the exterior and the intestinal tract of the birds transported in these crates. This can explain why different flocks from different farms harbored the same genotype in their intestines. As in this study, the *Campylobacter* genotypes isolated from the crops and the duodena differ from these isolated from the ceca. It is possible that the strains in the upper part of the intestinal tract originated from recent contamination. However, this hypothesis requires further investigation.

CHAPTER IV

Investigation of the concurrent colonization of poultry flocks with *Campylobacter* and *Salmonella* and assessment of the sampling site for status determination at the slaughterhouse

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Summary

The prevalence of *Campylobacter* and *Salmonella* colonized flocks at slaughter age was determined and an association between the concurrent colonization with these two pathogens was investigated. Furthermore, the best sampling site for status determination at the slaughterhouse was evaluated. Fifty-six broiler flocks and 20 spent laying hen and breeder flocks were sampled in six slaughterhouses. Samples were taken from three different sites of the gastrointestinal tract, namely from the crop, the duodenum and the ceca.

Of the broiler flocks, 73% were *Campylobacter* positive, whereas 13% were colonized with *Salmonella* at the moment of slaughter. Concerning the laying hen and breeder flocks, all flocks were colonized with *Campylobacter* and 65% of the flocks were *Salmonella* positive. No association was found between *Campylobacter* and *Salmonella* prevalence within broiler flocks. Since all laying hen and breeder flocks were *Campylobacter* colonized, no association between the concurrent colonization of both pathogens could be determined. At slaughterhouse level, sampling only the duodena was sufficient to determine the *Campylobacter* status, whereas a combination of the three sampling sites was necessary to detect all *Salmonella* colonized flocks.

1. INTRODUCTION

Campylobacter and *Salmonella* are the two most important causes of human bacterial gastroenteritis in the industrialized world. Poultry products are an important source in the transmission to humans for both zoonoses. Studies have shown that the consumption of contaminated chicken meat is responsible for 20% to 40% of human campylobacteriosis cases, and 20% of human salmonellosis cases (Van Pelt *et al.*, 1999; Nadeau *et al.*, 2002; Vellinga and Van Loock, 2002).

At slaughter age, the intestinal content of chickens may harbor *Campylobacter*, *Salmonella*, or both, and during the different stages of processing the intestinal content can contaminate the poultry meat (Oosterom *et al.*, 1983; Izat *et al.*, 1988; Lillard, 1990). Many studies have determined the prevalence of broiler flocks colonized with *Campylobacter*, *Salmonella,* or both, at slaughter age. The reported prevalence ranges from 3% in Finland to more than 90% in the UK for *Campylobacter*, and from 0% in Sweden to 77% in Canada for *Salmonella* (Renwich *et al.*, 1992; Wierup *et al.,* 1995; Evans and Sayers, 2000; Perko-Mäkelä *et al.*, 2002). Only a few studies have investigated the possible association between *Campylobacter* and *Salmonella* prevalence within poultry flocks. Though no association was reported in a Danish study (Wedderkopp *et al.,* 2001), a positive correlation was found in the studies of Jacobs-Reitsma *et al.* (1995) and Jacobs-Reitsma (1995). In these Dutch studies *Campylobacter*-free flocks were more often also *Salmonella* free, and *Campylobacter*-positive flocks were more often also positive for *Salmonella.*

The intestines, especially the ceca are the primary sites of colonization for *Campylobacter* as well as for *Salmonella* (Fanelli *et al.*, 1970; Beery *et al.*, 1988; Achen *et al.*, 1998). Just before slaughter, birds are subjected to feed withdrawal during transport to the slaughterhouse, resting time and sometimes even a few hours before transport. Some studies demonstrated that feed withdrawal in market-age broilers resulted in an increased incidence of *Salmonella*-positive crops and, less pronounced, in *Salmonella*-positive ceca. The number of *Salmonella*-positive crops may even exceed the number of positive ceca (Hargis *et al.*, 1995; Ramirez *et al.*, 1997; Corrier *et al.*, 1999). Byrd *et al.* (1998) showed that following feed withdrawal significant more crops were *Campylobacter* contaminated than ceca. Therefore, it is possible that the ceca are not the best choice for determining the *Campylobacter* and *Salmonella* prevalence in chickens at the slaughterhouse. However, the studies above are

performed on individual birds, so it should be investigated if the results reported can be extrapolated to the flock level.

The study aimed to determine the prevalence of broiler flocks and laying hen and breeder flocks with *Campylobacter* and *Salmonella* at slaughter age; to investigate an association between the concurrent colonization of flocks with *Campylobacter* and *Salmonella*; and to evaluate the best sampling site for determining the *Campylobacter* and *Salmonella* prevalence in flocks at slaughterhouse level.

2. MATERIALS AND METHODS

Sampling. During the period from January 2002 to March 2004, 76 flocks were examined for *Salmonella* and *Campylobacter* carriage[2]. A flock was defined as all birds reared in the same poultry house on a farm. The sampled flocks consisted of 56 broiler flocks and 20 spent laying hen and breeder flocks. Flock size ranged between 1100 and 18000 chickens. Broiler flocks were between 35 and 42 days old when slaughtered, whereas the laying hen and breeder flocks were between 10 and 26 months old. The broiler flocks were slaughtered in four Belgian slaughterhouses, whereas the other flocks were slaughtered in three (other) Belgian slaughterhouses. The flocks were sampled just before slaughter and during processing. From each flock, 30 living birds were randomly chosen from 15 crates from different transport containers. Of each bird, a swab sample of the crop was taken. In addition, 30 gastrointestinal tracts per flock (95% CI to detect a prevalence of 10% for more than 1000 birds) were collected at the slaughter line just after mechanical evisceration. All samples were packed in sterile plastic bags, transported to the laboratory under cooled conditions and processed the same day.

Bacterial culture for *Salmonella*. From each of the 30 gastrointestinal tracts, 1 g content of the duodenum and 1 g content of one cecum were aseptically collected. These samples were pooled, resulting in three subsamples of 10 g ceca content and three subsamples of 10 g duodenum content. The pooled samples were homogenized with 90 ml of buffered peptone water (BPW, Oxoid CM509, Basingstoke, UK) in a stomacher blender at normal speed. The 30 crop swabs were pooled to three subsamples and homogenized with 25 ml of BPW each. The homogenates were incubated at 37°C for 16 to 20 h. From all pre-enrichment broths, 100

[2] The flocks are the same as in Chapter II (+ 4 extra flocks)

µl was plated onto diagnostic semi-solid *Salmonella* agar (Diassalm, LabM 537, Lancashire, UK) and 100 µl was added to 10 ml Rappaport-Vassiliadis bouillon (RV, Oxoid CM669, Basingstoke, UK). After incubation for 24 h at 42°C, a loopful of *Salmonella* suspected Diassalm plates and 10 µl of all RV tubes were plated onto xylose lysine deoxycholate plates (XLD, Oxoid CM469, Basingstoke, UK) and incubated at 37°C for 24 h. Presumptive *Salmonella* colonies were confirmed at genus level by PCR using the primers described by Aabo *et al.* (1993). The reaction mixture and amplification protocol were as described by Botteldoorn *et al.* (2003). Per flock, one *Salmonella* isolate per pooled sample was further characterized, which resulted in a maximum of 9 isolates per flock if all plates showed *Salmonella* growth. The *Salmonella* isolates were clustered by rep-PCR as described by Rasschaert *et al*. (2005). A few randomly chosen isolates per cluster were sent to the Belgian reference laboratory for *Salmonella* for serotyping according to the Kauffmann-White scheme (Popoff and Le Minor, 1997).

Bacterial culture for *Campylobacter*. Of each of the BPW homogenates, 1 ml was added to 9 ml selective Preston broth (nutrient broth n°2 Oxoid CM 67, Basingstoke UK, enriched with 5% (vol/vol) lysed defibrinated horse blood and 1% Preston Supplement (5000 IU polymixin B, 0.010 g rifampicin, 0.0076 g trimethroprim and 0.010 g amphotericin dissolved in 10 ml ethanol)). The enrichment broths were incubated for 24 to 48 h at 42°C under microaerobic conditions (6% CO_2, 6% H_2, 4% O_2 and 84% N_2). After 24 h, 10 µl of each enrichment broth was plated onto modified cefoperazone charcoal deoxycholate agar (mCCDA; Oxoid CM 739 plus SR155, Basingstoke, UK) and incubated at 42°C under microaerobic conditions. If there was no *Campylobacter* growth on mCCDA after 24 h of incubation, 10 µl of the 48-h incubated enrichment broth was plated on a new mCCDA plate. Morphologically typical colonies were picked, examined by Gram-staining and subcultured on a blood agar plate (CM 965 and L13, Oxoid, Basingstoke, UK and 5% (v/v) defibrinated horse blood) and stored in whole horse blood at –80°C for further examination. Per flock, one *Campylobacter* isolate per pooled sample was stored, which resulted in a maximum of 9 isolates per flock if all plates showed *Campylobacter* growth. Identification on species level was performed according to the PCR assay of Vandamme *et al.* (1997) for *C. jejuni* and *C. coli*, and according to the PCR assay of Linton *et al.* (1996) for *C. lari.*

A flock was considered *Salmonella* or *Campylobacter* colonized, when *Salmonella* or *Campylobacter*, respectively, was detected in at least one of the pooled samples of the crop, the duodenum or the ceca.

Statistical analysis. The chi square (χ^2) test was used to investigate the association between *Campylobacter* and *Salmonella* prevalence within poultry flocks, and to determine the differences in *Campylobacter* and *Salmonella* colonization of the crops, the duodena and the ceca. Differences were considered to be nonsignificant at $p \geq 0.05$. When the data were not suitable for the χ^2 test, the Cramers' V test was used.

3. RESULTS

Broiler flocks. Forty one (73%) flocks were colonized with *Campylobacter*, whereas 7 flocks (13%) harbored *Salmonella* in the gastrointestinal tract at the moment of slaughter. Three flocks were positive for both *Campylobacter* and *Salmonella* at the moment of slaughter, and 11 flocks were negative for the two species (Table 1). There was none to little association between *Campylobacter* and *Salmonella* occurrence within broiler flocks (Cramers'V=0.26). Among the 41 *Campylobacter*-positive flocks, 32 flocks were colonized with only one *Campylobacter* species: 29 flocks with *C. jejuni*, two flocks with *C. coli* and one flock with *C. lari*. Six flocks carried two species in the intestines: four flocks harbored *C. jejuni* and *C. coli* in the gastrointestinal tract, whereas the other two were colonized with *C. jejuni* and *C. lari*. The isolates of the three remaining flocks were not identified on species level. The seven *Salmonella*-positive flocks were all colonized with only one *Salmonella* serotype: two flocks were colonized with *Salmonella* Typhimurium O5+, two flocks with *Salmonella* Infantis, whereas the other three flocks were colonized with *Salmonella* London, *Salmonella* Hadar and an isolate which was not typeable, respectively.

The site of colonization for the *Campylobacter*- and *Salmonella*-positive flocks is shown in Table 2. Concerning the *Campylobacter* colonized flocks, there was a significant difference between the prevalence of *Campylobacter* in the three sampling sites (χ^2 test, p=0.038). More flocks were colonized in the duodena and ceca than in the crops. On the other hand, there was no association between the prevalence of *Salmonella* in the three sampling sites (Cramers'V=0.040). Sampling only the duodenum would have detected all *Campylobacter*-positive flocks, whereas all three sampling sites had to be sampled to identify all flocks colonized with *Salmonella* (Table 2).

Table 1. The distribution in the prevalence of *Campylobacter* and *Salmonella* colonized flocks

broiler flocks			
	Campylobacter +	*Campylobacter* -	total
Salmonella +	3	4	7
Salmonella -	38	11	49
Total	41	15	56
laying hen and breeder flocks			
	Campylobacter +	*Campylobacter* -	total
Salmonella +	13	0	13
Salmonella -	7	0	7
Total	20	0	20

Table 2. The distribution of *Campylobacter* and *Salmonella* prevalence in the crop, duodenum and ceca

	crop	**duodenum**	**ceca**	**crop +duodenum**	**duodenum +ceca**	**crop +ceca**	**total**
broilers flocks							
Campylobacter +	28 (68%)	41 (100%)	36 (88%)	41 (100%)	41 (100%)	39 (95%)	41
Salmonella +	3 (43%)	3 (43%)	2 (29%)	6 (86%)	4 (57%)	5 (71%)	7
laying hen and breeder flocks							
Campylobacter +	13 (65%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)	20
Salmonella +	6 (46%)	10 (77%)	7 (54%)	12 (92%)	12 (92%)	10 (77%)	13

Laying hen and breeder flocks. The 20 sampled flocks were all colonized with *Campylobacter*, and 13 (65%) of the flocks harbored *Salmonella* in the gastrointestinal tract at slaughter (Table 1). Since all flocks were colonized with *Campylobacter*, it was not possible to determine an association between *Campylobacter* and *Salmonella* occurrence in flocks. Four flocks were colonized with only one *Campylobacter* species (*C. jejuni*), 11 flocks were colonized with two species (*C. jejuni* and *C. coli*), and from five flocks three species were isolated (*C. jejuni*, *C. coli* and *C. lari*). From 11 of the 13 *Salmonella*-positive flocks, *Salmonella* Enteritidis was isolated. The two remaining flocks were colonized with *Salmonella* Braenderup and a strain which was not typeable by serotyping, respectively.

There was a weak association between the prevalence of *Campylobacter* in the three sampling sites (Cramers'V=0.51). As for the broiler flocks, the duodena and the ceca were more often colonized than the crops. Sampling the duodena or the ceca would have detected all *Campylobacter*-positive flocks. Concerning *Salmonella*, there was no significant difference between the prevalence in the three sampling sites ($\chi 2$ test, p=0.333). As was found for the

broiler flocks, all three sampling sites were necessary to detect all *Salmonella* colonized flocks.

4. DISCUSSION

In the present study, 73% of the broiler flocks and 100% of the laying hen and breeder flocks were colonized with *Campylobacter*. The proportion of European broiler flocks colonized with *Campylobacter* at slaughter age varies among countries, ranging from 3% in Finland (Perko-Mäkelä *et al.*, 2002) to more than 90% in the UK (Evans and Sayers, 2000). In a Belgian study of Herman *et al.* (2003), 67% of the broiler flocks were at the time of slaughter *Campylobacter* colonized which is in agreement with the prevalence found in the present study. Limited data is available about the *Campylobacter* prevalence in laying hen and breeder flocks. Jacobs-Reitsma (1995) found a prevalence of 67% in breeder flocks. It is generally accepted that for broiler flocks, the birds remain infected until the slaughter age of 6 weeks (Newell and Wagenaar, 2000). However, after 8 weeks, the number of infected birds and the level of campylobacters recoverable may gradually reduce (Achen *et al.*, 1998). Self-limitation of infection has also been reported in for example gulls, which became *Campylobacter* free within a period of 4 weeks (Glunder *et al.*, 1992). However, instead of reduced colonization, the present study revealed that colonization in laying hen and breeder flocks is higher than in broiler flocks in terms of the number of flocks colonized and the number of species isolated from the gastrointestinal tract.

In contrast to *Campylobacter*, the *Salmonella* status of flocks can change from positive to negative in the period between status determination and processing the flock. For example, in a Belgian study in which 18 broiler flocks were followed from hatching to the slaughterhouse, 10 flocks received a *Salmonella*-positive status during rearing. However, the number of positive flocks dropped to 6 at slaughter age (Heyndrickx *et al.*, 2002), which shows the importance of determining the *Salmonella* colonized flocks at the time they enter the processing line. In the present study, 13% of the broiler flocks were colonized with *Salmonella*. This is comparable to the results of a recently published Dutch study, in which approximately 12% of the broiler flocks at slaughter age were *Salmonella* colonized on the farm (van de Giessen *et al.*, 2006). This is a decline compared to an earlier Belgian study of Heyndrickx *et al.* (2002) and the earlier Dutch study of Jacobs-Reitsma *et al.* (1995) which

reported *Salmonella* colonized flock prevalence of 33% on the farm at slaughter age and 27% in the slaughterhouse, respectively. In the present study, 65% of the laying hen and breeder flocks were *Salmonella* colonized. In execution of regulation EC/2160/2003 on the control of *Salmonella* and other specified foodborne zoonotic agents, the E.U. have recently published a report about the *Salmonella* prevalence in laying hen flocks during the last 9 weeks of their production period in 2004-2005. The prevalence ranged from 0% in Luxembourg to 80% in Portugal. The prevalence in Belgium (35%) was almost half of the reported prevalence in the present study (EFSA, 2006a). However, the prevalence was determined by collecting fecal and environmental samples during the last 9 weeks of the production period of the flocks. Possible explanations for the higher prevalence reported in the present study are that the birds acquire a new infection during the last weeks of their production period or even during transport as demonstrated by Rigby and Pettit (1980). The birds can also change from *Salmonella* carriers to shedders for example during transport which is known to be stressful (Rigby and Pettit, 1980).

In the present study, no positive or negative association was found between *Campylobacter* and *Salmonella* prevalence within broiler flocks. This in agreement with the results of Wedderkopp *et al.* (2001) but in contrast with the outcome of the study of Jacobs-Reitsma *et al.* (1995). In the study of Jacobs-Reitsma *et al.* (1995) a positive correlation was found between *Campylobacter* and *Salmonella* contamination within a broiler flock. *Campylobacter*-free flocks were more often also *Salmonella* free, and *Campylobacter*-positive flocks were more often also positive for *Salmonella.* Jacobs-Reitsma (1995) reported also a positive correlation within breeder flocks. However, since all laying hen and breeder flocks were *Campylobacter* colonized in the present study, no correlation could be demonstrated.

In the present study, the duodenum was most often *Campylobacter* or *Salmonella* colonized, followed by the ceca and finally the crop. This in contrast with the studies of a research group (Hagris *et al.*, 1995; Ramirez *et al.*, 1997; Corrier *et al.*, 1999) which has demonstrated that feed withdrawal in market-age broilers resulted in an increased incidence of *Salmonella*-positive crops and less pronounced in *Salmonella* positive ceca. The number of *Salmonella*-positive crops may even exceed the number of positive ceca. Byrd *et al.* (1998) observed the same for *Campylobacter* colonized flocks. However, these studies are performed on individual birds, whereas the present study is performed on flock level, which is a possible

explanation for the different outcome. It is remarkable in the present study that for the broiler flocks as well as the laying hen and breeder flocks, sampling only the duodenum was sufficient to detect all *Campylobacter* colonized flocks, whereas for identifying all *Salmonella* colonized flocks a combination of the three sites was necessary.

In conclusion, the present study has demonstrated that there was no association between *Campylobacter* and *Salmonella* concurrent colonization. Sampling only the duodenum has been shown to be sufficient to determine the *Campylobacter* prevalence in the poultry flocks at the slaughterhouse level. However, for epidemiological studies in which *Campylobacter* isolates are characterized, it is still necessary to take samples of the three sampling sites, since the strains from crop, duodenum and cecum may differ as demonstrated by Rasschaert *et al.* (2006). For determining the *Salmonella* prevalence in poultry flocks at the slaughterhouse level, the three sites have to be sampled.

CHAPTER V

Impact of the slaughter line contamination on the presence of *Salmonella* on broiler carcasses

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Summary

The aim of the study was to assess the impact of *Salmonella* present on the slaughter line before processing on broiler carcass contamination. Three Belgian broiler slaughterhouses were each visited twice. Samples were taken from the slaughter line after the cleaning and the disinfection process and before slaughter of the first flock. During the slaughter of the first flock, feathers and neck skins were collected at various points of the slaughter process. Swab samples were also taken from the crates in which the birds were transported. In two slaughterhouses, the slaughter line was contaminated with *Salmonella* before the onset of slaughter, especially the shackles, conveyer belt and the plucking machine in the dirty zone. During slaughter, the carcasses of the first *Salmonella*-free flock became contaminated with the same strains as isolated previously from the slaughter line. Implementation of logistic slaughter is only successful when the cleaning and disinfection process completely eliminates the *Salmonella* contamination of the slaughter line. Only if this is achieved, will the slaughter of *Salmonella*-free flocks result in the absence of *Salmonella* on the carcasses after slaughter.

1. INTRODUCTION

Salmonella enterica subsp. *enterica* is one of the major foodborne causes of gastroenteritis in most industrialized countries. In Belgium, 9543 *Salmonella* isolates from human infections were sent in 2004 for further characterization to the National Reference Centre for *Salmonella* and *Shigella* (NRSS, 2004). The serotypes mostly isolated in 2004 were *Salmonella* serotype Enteritidis (64%) and *Salmonella* serotype Typhimurium (26%) (NRSS, 2004). According to Van Pelt *et al.* (1999), eggs and poultry meat are responsible for 39% and 21% of human salmonellosis cases, respectively, whereas human salmonellosis is caused by pork in 25% of the cases and by beef in about 10% of the cases. Contamination of poultry products can occur through the whole production chain, but until now, most studies have been focusing on the primary production. Several risk factors for *Salmonella* contamination have already been identified in the farm, such as vertical transmission from breeder flocks to their offspring, contamination of equipment in the hatchery, a poor level of hygiene in the farm, the presence of rodents and insects on the farm, inadequate cleaning between rotation of flocks, and contamination of the feed and drinking water (Davies and Wray, 1995; Davies and Wray, 1996; Davies *et al.*, 1997; Rose *et al.*, 1999; Davies and Breslin, 2003; Doyle and Erickson, 2006). Several control measures have been implemented to reduce *Salmonella* contamination of poultry flocks at farm level such as vaccination of the breeder flocks, application of competitive exclusion, the use of prebiotics, acidification of feed and water and strict hygiene measures on the farm (Doyle and Erickson, 2006). At slaughter age, it is important to maintain the birds *Salmonella* free during transport and slaughter. However, transport in inadequately cleaned and disinfected containers (Rigby *et al.,* 1980), cross-contamination by the slaughter environment or by *Salmonella* contaminated flocks to the carcasses of *Salmonella*-free flocks are identified as possible risk factors at this stage of production (Corry *et al.,* 2002; Olsen *et al.*, 2003). To reduce cross-contamination, logistic slaughter has been applied since 1999 in Belgium. Flocks with a *Salmonella*-free status are slaughtered first followed by *Salmonella*-positive flocks. The *Salmonella* status is determined by collecting fecal material in the broiler house using two pairs of overshoes within three weeks before slaughter. In a previous Belgian study, 18 broiler flocks were followed from hatching to slaughter. Though eight flocks had a *Salmonella*-free status, the carcasses of seven of these flocks were contaminated with *Salmonella* after slaughter while four of these flocks were

slaughtered first on the sampling days (Heyndrickx *et al.,* 2002). This may indicate that the *Salmonella* contamination originated from the slaughter environment.

The aims of the present study were first to assess the presence of *Salmonella* on the slaughter line before processing the first flock, and second, to determine the impact of *Salmonella* present on the slaughter line on the carcass contamination of the first flock slaughtered.

2. MATERIALS AND METHODS

The poultry processing plants. The study was conducted in three Belgian broiler slaughterhouses (A,B and C[3]) from June to November 2005. The slaughterhouses were visited on a Tuesday or a Wednesday after at least one day of operation in the week. Each slaughterhouse was visited twice with a minimum interval of three weeks between visits. A similar slaughter procedure was applied in the three slaughterhouses. In the living area, the birds were unloaded, hanged manually, electrically stunned and killed. In a second separated area, the birds were scalded in a counter current flow scalding tank at a temperature of ± 51°C before they were mechanically plucked. The heads of the birds were removed before the carcasses were hung over on the evisceration line. Finally, the mechanically evisceration took place in a third room. During processing, only potable water was used in the three slaughterhouses. Plants A, B and C had processing capacities of 9000, 6000 and 6000 birds per hour, respectively. Slaughterhouse A was the only slaughterhouse with two killing lines, but only one killing line was included in the sampling plan.

Sample collection. An overview of the sampled slaughter equipment and the samples taken from the first poultry flock slaughtered at each visit are shown in Table 1. The samples from the slaughter line were taken one hour before the slaughter activities started and several hours after the cleaning and disinfection process had ended. All samples, except the water scalding samples, consisted of one swab moistened with sterile peptone water (0.1%). From each scalding tank, different water samples (25 ml) were collected before and during slaughter. During slaughter of the first flock, feathers from the breast and the wings were collected while the birds were hanging on the shackles before scalding, after scalding and from the plucking machine. Thirty neck skin samples were collected immediately after plucking and 30 neck

[3] Slaughterhouses A, B and C are the same slaughterhouses as A, B and C in Chapters II and III.

skin samples after evisceration. Up to three neck skins were pooled to obtain a sample of at least 25 g. All samples collected during processing of the first flock were taken at evenly distributed intervals over the time needed to process the complete flock. The six flocks had all received a *Salmonella*-negative status as stated on the transport documents. To check this status at the moment of slaughter, 60 gastrointestinal tracts from each flock were collected just after evisceration (95% CI to detect a prevalence of 5% in a flock). Furthermore, six containers used to transport the flock were sampled just before the crates were washed and disinfected. Of each transport container, four samples were taken (ca. 400 cm^2) with four swabs and pooled to one sample. All samples were transported to the laboratory under cooled conditions and processed immediately.

Table 1. Overview of the number of samples taken in the three slaughterhouses before and during processing

Samples	Number of samples	Samples	Number of samples
Hanging area before processing		**Evisceration before processing**	
3 shackles before the hanging area	1	3 shackles	3
2 wheels and 25 cm conveyer belt before the hanging area	1	2 wheels and 25 cm conveyer belt	1
3 shackles after the hanging area	1	neck breaker - 1 element	1
2 wheels and 25 cm conveyer belt after the hanging area	1	vent cutter - 1 element	1
3 shackles after stunning	1	abdominal cavity opening machine - 1 element	1
2 wheels and 25 cm conveyer belt after stunning	1	scoops - 1 element	1
		cropper - 1 element	1
Scalding tank before processing		neck cutter - 1 element	1
3 shackles	3	neck remover - 1 element	1
2 wheels and 25 cm conveyer belt	3	neck skin cutter - 1 element	1
doors (400 cm^2)	3	lung remover - 1 element	1
roof (400 cm^2)	3	inside/outside bird washer - 1 element	1
just above the water surface (10 cm^2)	3		
25 ml scalding water	4	**Samples during processing the first flock**	
		feathers before scalding (25g)	3
Plucking machine before processing		feathers after scalding (25g)	3
3 shackles	3	feathers from the plucking machine (25g)	3
2 wheels and 25 cm conveyer belt	3	25 ml scalding water	6
plucking fingers – 1 element	3	neck skins after plucking (25g)	30
plastic bands between fingers (400 cm^2)	3	neck skins after evisceration (25g)	30
construction (400 cm^2)	3	4 crates of a transport container (4x400 cm^2)	6
		pooled sample of 10 duodena	6
		pooled sample of 10 ceca	6

Bacteriological examination. Twenty-five ml of each water sample was mixed with 25 ml of double-strength buffered peptone water (BPW, Oxoid CM509, Basingstoke, UK). Forty ml of the pre-enrichment media BPW was added to all swab samples, except for the swabs of the transport containers to which 100 ml of BPW was added before homogenizing in a stomacher blender at normal speed. Twenty-five grams of the feather samples was mixed with 225 ml of BPW. Each neck skin sample (25 g) was stomachered in 225 ml of BPW. From each of the 60 gastrointestinal tracts, 1 g of the duodenum and 1 g of the cecum were aseptically collected. These samples were pooled to create six subsamples of 10 g ceca content and six subsamples of 10 g duodenum content. These subsamples were homogenized with 90 ml of BPW in a stomacher blender.

After incubation of the pre-enrichment media at 37°C for 18 h, 100 µl was plated onto diagnostic semi-solid *Salmonella* agar (Diassalm, LabM 537, Lancashire, UK) and 100 µl was added to 10 ml Rappaport-Vassiliadis bouillon (RV, Oxoid CM669, Basingstoke, UK). After incubation for 24 h at 42°C, a loopful from the edge of the purple migration zone from the Diassalm plates was plated onto xylose lysine deoxycholate (XLD, Oxoid CM469, Basingstoke, UK). If the plates showed a large migration zone (complete discolloration), two loopfuls of the zone were plated on two XLD plates. Ten µl of each RV tube was plated on XLD. All XLD plates were incubated at 37°C for 24 h. From the XLD plates streaked out from the RV tubes, two morphologically typical colonies were picked. That way, a maximum of four colonies per sample were further examined. Presumptive *Salmonella* colonies were confirmed at genus level by PCR using the primers described by Aabo *et al.* (1993). The reaction mixture and amplification protocol were as described by Botteldoorn *et al.* (2003).

Characterization of the *Salmonella* isolates. All *Salmonella* isolates were characterized by enterobacterial repetitive intergenic consensus (ERIC) PCR as previously described by Rasschaert *et al.* (2005). ERIC-PCR can be used to limit the number of strains that have to be serotyped as different strains belonging to the same serotype cluster together at a delineation level of 95%. At least two isolates per cluster were subsequently serotyped by the Belgian *Salmonella* reference laboratory. Randomly selected isolates of each serotype were characterized at strain level by pulsed-field gel electrophoresis (PFGE). The isolates were grown for 18 h on tryptone soya agar (TSA, Oxoid CM0131, Basingstoke, UK) at 37°C. The cells were suspended in cold Pett IV buffer (1M NaCl, 10mM Tris-HCl pH8, 10mM Na_2EDTA) and adjusted to an OD_{600} value of 0.8. The method of Olsen *et al.* (1994) was

followed for preparing the plugs. Plug slices were digested for 18 h with 30 U of *Xba*I and *Not*I (Invitrogen, Paisley, UK) in single digestion reactions. DNA fragments were separated by Chefmapper in a 1% Seakem agarose gel (Biowhittaker Molecular Applications, Rockland, Maine, USA). The running conditions were 6 V/cm at 14°C in 0.5 x TBE buffer for 22 h with a ramping time from 4 to 40 s for the *Xba*I enzyme or 24 h with a ramping time from 2 to 12 s for the *Not*I enzyme. PFGE profiles were clustered with GelCompar 3.0 (Applied Maths, Sint-Martens-Latem, Belgium) using the Dice coefficient (1% position tolerance) and the unweighted-pair group method using arithmetic averages algorithm (UPGMA). A PFGE genotype was assigned on the basis of a difference in the absence or presence of at least one band in at least one of the two profiles (*Xba*I and *Not*I). Genotypes within serotypes were indicated by the capital of the name of the serotype followed by a number (e.g. *Salmonella* Montevideo genotype 1 is idicated as M1). A small shift of one band in maximum one of the two profiles was indicated by an apastrophe.

***Salmonella* isolates from flocks slaughtered in the week before the sampling day.** As there is a legal obligation to determine the *Salmonella* status of all Belgian broiler flocks before slaughter, the slaughterhouses were able to give an overview of all *Salmonella*-positive flocks slaughtered in the week before the sampling days. Some isolates from these *Salmonella*-positive flocks could be recuperated from the laboratories to which the overshoes were sent to determine the *Salmonella* status. These isolates were characterized by ERIC-PCR and PFGE as described above.

3. RESULTS

In total, 881 samples were collected in the three slaughterhouses. Two hundred and twenty-one samples were *Salmonella* positive (25%) and 553 *Salmonella* isolates were further characterized.

Slaughterhouse A

The first sampling day

On the first sampling day in slaughterhouse A, 17 out of 50 samples (34%) taken from the slaughter line were contaminated with *Salmonella*. Nine genotypes belonging to six serotypes were found on the slaughter equipment (Table 2). *Salmonella* Typhimurium O5+ genotype T1 and *Salmonella* Paratyphi B genotype P1 were most commonly isolated from the slaughter environment. In the plucking and scalding area, the plucking machine was the most contaminated. The plastic bands (between the rows of the plucking fingers) in the plucking machine were contaminated with seven different genotypes.

The *Salmonella*-free status of the first slaughtered flock was confirmed by the absence of *Salmonella* in the intestines. Nevertheless, the transport crates, the feathers before and after scalding, the feathers collected during plucking and the neck skins samples after plucking and after evisceration were contaminated with *Salmonella.* These samples were contaminated with the same strains as previously isolated from the slaughter line before processing, except *Salmonella* Minnesota genotype Mi1 which was isolated from ten neck skins samples after evisceration. Only *Salmonella* Indiana strain I1 which was isolated from different places from the slaughter line was not found during slaughter of the first flock (Table 2).

In the week before the first sampling day, two flocks with a *Salmonella*-positive status were slaughtered. A flock slaughtered four days before the sampling day was colonized with *Salmonella* Typhimurium O5+ strain T1.

The second sampling day

On the second sampling day in slaughterhouse A, 23 out of 56 samples (41%) of the slaughter line were contaminated with *Salmonella*. The shackles and wheels were the most contaminated. Only in the evisceration room were the shackles and wheels *Salmonella* free. Seven genotypes belonging to five serotypes were found in the slaughter environment (Table 2). Although this flock had a *Salmonella*-free status, two strains were isolated from the duodenal content of this flock: *Salmonella* Paratyphi B strain P5 and *Salmonella* Typhimurium O5+ strain T1. The former was not found during slaughter of the flock, whereas the latter was found on the slaughter line before slaughter and during slaughter of the first flock and was also frequently isolated on the first sampling day. *Salmonella* Blockley strain B1, *Salmonella* Minnesota strain Mi1, *Salmonella* Montevideo strain M1 were also found on both sampling days (Table 2). Again, the crates, the feather samples and the neck skin samples were contaminated with the same strains as previously isolated from the slaughter line (Table 2).

Four flocks with a *Salmonella*-positive status were slaughtered in the week before the second sampling day. Two flocks slaughtered five and six days before the second sampling day were colonized with *Salmonella* Typhimurium O5+ strain T1. These two flocks and the flock from which the same strain was isolated in the week before the first sampling day originated all from the same farm.

Slaughterhouse B

The first sampling day

On the first sampling day, only four of the 54 samples (7%) taken from the slaughter line were *Salmonella* positive (Table 3). The *Salmonella*-negative status of the flock was confirmed by the absence of *Salmonella* in the intestines. During processing the flock, *Salmonella*-positive samples were collected from the feathers before scalding, the scalding water and five neck skin samples (Table 3). All isolates belonged to *Salmonella* Livingstone genotype L1. Two days before the first sampling day, one *Salmonella*-positive flock was slaughtered. No isolates of this flock were available.

The second sampling day

On the second sampling day, 23 of the samples (43%) taken from the slaughter equipment were *Salmonella* positive (Table 3). Especially the scalding tank, inclusive of the scalding water was contaminated with *Salmonella*. Only two strains were isolated from the slaughterhouse equipment: *Salmonella* Indiana strain I2 and *Salmonella* Virchow strain V1. No *Salmonella* was isolated from the intestines of the flock slaughtered first, though *Salmonella* Indiana strain I2 was isolated from one crate. The feathers after scalding and during plucking, and 29 of the neck skin samples after plucking and 15 neck skin samples after evisceration were contaminated with *Salmonella*. The same two strains were isolated from the skin samples as from the slaughter line before processing. *Salmonella* Agona strain A3 was found on the feathers collected from the plucking machine and on the neck skins after plucking, but was not isolated from the slaughter line before processing.

Two *Salmonella*-positive flocks were slaughtered eight and six days before the second sampling day. These two flocks originated from the same farm and harbored *Salmonella* Virchow strain V2 in the intestines. On the day before the second sampling day, a flock colonized with *Salmonella* Agona strain A3 was slaughtered.

Slaughterhouse C

No *Salmonella* was isolated on the first sampling day in slaughterhouse C. On the second sampling day, only two neck skin samples after plucking and one neck skin sample after evisceration were contaminated with *Salmonella* Livingstone strain L2. In the week before the first sampling day, no flocks with a known positive *Salmonella* status were slaughtered. However, a few foreign flocks were slaughtered for which the status was not determined. In the week before the second sampling day, one *Salmonella*-positive flock and some flocks with an unknown status were slaughtered.

Table 2. *Salmonella* contaminated samples on the slaughter line and during processing of the first flock in slaughterhouse A

	first sampling day			second sampling day		
	n°	**serotype**	**genotype**	**n°**	**serotype**	**genotype**
Cleaned slaughter equipments						
hanging area						
shackles before the hanging area	-			1/1	Paratyphi B	P2
					Kentucky	K1
wheels and conveyer belt before the hanging area	-			1/1	Paratyphi B	P2
					Blockley	B1
shackles after the hanging area	-			1/1	Paratyphi B	P2
wheels and conveyer belt after the hanging area	-			1/1	Paratyphi B	P2
shackles after stunning	-			1/1	Paratyphi B	P2
wheels and conveyer belt after stunning	-			1/1	Paratyphi B	P2
					Blockley	B1
scalding tank						
shackles	2/3	Typhimurium O5+	T1	1/3	Typhimurium O5+	T1
		Blockley	B1		Blockley	B1
		Paratyphi B	P1			
wheels and conveyer belt	1/3	Typhimurium O5+	T1	3/3	Typhimurium O5+	T1
					Paratyphi B	P3
					Blockley	B1
doors	1/3	Typhimurium O5+	T1	0/3		
roof	1/3	Typhimurium O5+	T1	1/3	Paratyphi B	P2
just above the watersurface	1/3	Indiana	I1	1/3	Paratyphi B	P2
plucking machine						
shackles	1/3	Paratyphi B	P1	2/3	Paratyphi B	P2
wheels and conveyer belt	3/3	Typhimurium O5+	T1	3/3	Typhimurium O5+	T1
		Paratyphi B	P1		Paratyphi B	P2
		Agona	A1		Blockley	B1
		Indiana	I1			
fingers	1/3	Paratyphi B	P1	1/3	Paratyphi B	P2
bands between fingers	3/3	Typhimurium O5+	T1	3/3	Paratyphi B	P2
		Paratyphi B	P1		Montevideo	M1’
		Agona	A2			M4
		Indiana	I1			
		Montevideo	M1			
			M2			
			M3			
construction	1/3	Paratyphi B	P1	0/3		
evisceration						
neck breaker	1/1	Paratyphi B	P1	0/1		
vent cutter	0/1			1/1	Paratyphi B	P2
					Blockley	B1
scoops	1/1	Typhimurium O5+	T1	1/1	Paratyphi B	P2
					Blockley	B1
First flock						
crates	1/6	Typhimurium O5+	T1’	2/6	Paratyphi B	P2
feathers before scalding	3/3	Typhimurium O5+	T1	3/3	Paratyphi B	P2
		Rissen			Blockley	B1
feathers after scalding	2/3	Typhimurium O5+	T1	3/3	Paratyphi B	P2
		Paratyphi B				
feathers from plucking machine	3/3	Typhimurium O5+	T1	3/3	Paratyphi B	P2
		Agona	A2		Blockley	B1
		Montevideo	M1		Typhimurium O5+	T1
					Montevideo	M1
						M4
scalding water during processing	0/6			1/6	Indiana	I1
neck skins after plucking	13/30	Typhimurium O5+ (5) *	T1	20/30	Typhimurium O5+ (3)	T1
		Paratyphi B (5)	P1		Paratyphi B (10)	P2
		Blockley (5)	B1			P3
					Blockley (5)	B1
					Montevideo (2)	M1
					Tennessee (1)	
neck skins after evisceration	18/30	Typhimurium O5+ (4)	T1	17/30	Typhimurium O5+ (1)	T1
			T2		Paratyphi B (14)	P2
		Paratyphi B (6)	P1			P2’
		Blockley (1)	B1			P4
		Minnesota (10)	Mi1		Blockley (3)	B1
					Minesota (1)	Mi1
duodenum	0/6			2/6	Paratyphi B	P5
					Typhimurium O5+	T1

*Within brackets is the number of isolates. On some occasions (see Materials and Methods), more than one colony was picked from a *Salmonella* suspected plate; therefore, the number of isolates exceeds the number of *Salmonella*-positive samples.

Table 3. *Salmonella* contaminated samples on the slaughter line and during processing of the first flock in slaughterhouse B

	first sampling day			second sampling day		
	n°	**serotype**	**genotype**	**n°**	**serotype**	**genotype**
Cleaned slaughter equipment						
scalding tank						
shackles	0/3			1/3	Indiana	I2
wheels and conveyer belt	1/3	Livingstone	L1	3/3	Indiana	I2
					Virchow	V1
doors	0/3			3/3	Indiana	I2
					Virchow	V1
roof	0/3			1/3	Indiana	I2
					Virchow	V1
just above the water surface	1/3	Livingstone	L1	2/3	Indiana	I2
					Virchow	V1
scalding water	0/4			2/4	Indiana	I2
					Virchow	V1
plucking machine						
wheels and conveyer belt	2/3	Livingstone	L1	3/3	Indiana	I2
					Virchow	V1
bands between fingers	0/3			2/3	Indiana	I2
					Virchow	V1
construction	0/3			2/3	Indiana	I2
evisceration						
wheels and conveyer belt	0/3			1/3	Indiana	I2
neck breaker	0/1			1/1	Indiana	I2
scoops	0/1			1/1	Indiana	I2
cropper	0/1			1/1	Indiana	I2
First flock						
crates	0/6			1/6	Indiana	I2
feathers before scalding	1/3	Livingstone	L1	0/3		
feathers after scalding	0/3			1/3	Indiana	I2
feathers from plucking machine	0/3			3/3	Indiana	I2
					Agona	A3
scalding water	1/6	Livingstone	L1	3/6	Indiana	I2
neck skins after plucking	4/30	Livingstone	L1	29/30	Indiana (24)*	I2
					Virchow (11)	V1
					Agona (4)	A3
neck skins after evisceration	1/30	Livingstone	L1	15/30	Indiana	I2

*Within brackets is the number of isolates. On some occasions (see Materials and Methods), more than one colony was picked from a *Salmonella* suspected plate; therefore, the number of isolates exceeds the number of *Salmonella*-positive samples.

4. DISCUSSION

Slaughtering broiler flocks colonized with *Salmonella* can lead to a contamination of both carcasses and slaughter line (Lillard, 1990; Corry, *et al.* 2002; Olsen, *et al.* 2003). The cleaning and disinfection process performed after the slaughter activities is expected to remove the existing *Salmonella* contamination from the slaughter environment. In the present study however, in two slaughterhouses the slaughter equipment was found to be still contaminated when slaughter activities started. In both slaughterhouses, the slaughter equipment in the plucking and scalding area was more contaminated than in the evisceration room. This may indicate that in the evisceration room the bacterial load is lower than in the plucking and scalding area or that the cleaning and disinfection process is more effective in the evisceration room.

Two strains, *Salmonella* Typhimurium strain T1 and *Salmonella* Blockley strain B1, were isolated from the slaughter line in slaughterhouse A on both sampling days. Some *Salmonella*-positive flocks, all colonized with the same *Salmonella* Typhimurium strain T1 and reared on the same farm, were slaughtered in the week before both sampling days. Slaughtering these flocks may be the source for the contamination of the slaughter equipment on both occasions. It is possible that strain B1 also re-entered the slaughterhouse, as it is a strain that circulates in Belgian flocks (unpublished data) or survived on the processing line. A flock colonized by *Salmonella* Agona strain A3 entered the slaughterhouse the day before the second sampling day in slaughterhouse B. This strain was not recovered from the slaughter line before slaughter, although it was isolated from the feathers collected from the plucking machine and carcasses after plucking. This observation indicated that this strain may have survived the cleaning and disinfection process but was not picked up by the sampling and isolation methods applied.

Slaughtering *Salmonella*-positive flocks can lead to a contamination of the slaughter line as demonstrated in slaughterhouse A. In slaughterhouse B however, the *Salmonella* strains (V2 and A3) from the positive flocks slaughtered before the second sampling day did not correspond with those found on the slaughter equipment before slaughter activities started. According to Olsen *et al.* (2003), some *Salmonella* strains can survive up to five days in the slaughter environment despite the daily cleaning and disinfection procedures. The results may

indicate that some *Salmonella* strains can better survive in the slaughter environment than others.

In the present study, 11% of the crates used to transport the flocks were contaminated with *Salmonella*, notwithstanding that the flocks were *Salmonella* negative. Even in the case that the flock was infected (slaughterhouse A, second sampling day) other *Salmonella* genotypes were isolated from the crates. Different studies have shown that the cleaning and disinfection process is often inadequate in eliminating *Salmonella* from crates. In the study of Rigby *et al.* (1982), 99% of the washed and disinfected crates examined were still contaminated with *Salmonella*. More recently, *Salmonella* was isolated from 13% to 87% of disinfected crates at eight Danish poultry slaughterhouses (Olsen *et al.*, 2003). According to Rigby *et al.* (1980) and Corry *et al.* (2002), more crates were contaminated with *Salmonella* after washing and disinfection than after unloading the birds. Even more, during this process, the crates may become contaminated with other *Salmonella* serotypes (Corry *et al.*, 2002). Rigby *et al.* (1980) have shown that the transport of broilers in *Salmonella* contaminated crates led to the contamination of the exterior of the birds. Therefore, the contamination of the feathers before scalding may have originated from the contaminated crates. This indicates that inadequately cleaned and disinfected crates can maintain a *Salmonella* contamination cycle during transport and slaughter.

During slaughter, *Salmonella* on the slaughter equipment can be spread out on the carcasses by the process water. This was demonstrated in the plucking machine, where most of the *Salmonella* strains found on the feathers collected during slaughtering were present on these machines before slaughter. Scalding and plucking in a contaminated environment resulted in contaminated carcasses leaving the dirty zone. The number of contaminated carcasses at this point in the slaughter process seemed to be related to the number of contaminated sampling points of the slaughter line as demonstrated on both sampling days in slaughterhouse B. The contamination of the evisceration line caused no further increase in the number of positive carcasses, even a reduction was observed on different occasions.

In slaughterhouse A, *Salmonella* Minnesota isolated from carcasses after evisceration was not found either from the environment or at the slaughter stage before. This serotype may have originated from the second scalding and plucking line as this line was not sampled during the investigation. On both sampling days, strains belonging to this serotype were genetically

undistinguishable indicating that this strain probably survived for a long time in this part of the slaughterhouse.

In conclusion, the separation in time between the slaughter of *Salmonella* infected and noninfected broiler flocks is a good control measure to prevent cross-contamination during processing. However, two conditions have to be fulfilled. First of all, the status must be determined correctly, which is difficult as the status is determined a few weeks before slaughter. In the time span between status determination and slaughter, the birds can become (apparently) clear of infection (Heyndrickx *et al.,* 2002) or the flock can acquire a new infection, e.g. during transport (Rigby and Pettit, 1980). There can also be an increased rate of shedding owing to the stressful transportation to the slaughterhouse (Corry *et al.,* 2002). Second, the slaughterhouse environment must be *Salmonella* free at the start of the day. The present study has demonstrated that contamination of the transport containers and the slaughter environment may lead to the contamination of the end product. Only the application of a daily cleaning and disinfection process which eliminate any *Salmonella* contamination can assure that the slaughter of *Salmonella*-free flocks when applying logistic slaughter will result in the absence of *Salmonella* on the carcasses after slaughter.

CHAPTER VI

External contamination of *Campylobacter*-free flocks after transport in cleaned and disinfected containers

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Adapted from

Rasschaert G., K. Houf, L. De Zutter (2007). External contamination of *Campylobacter*-free flocks after transport in cleaned and disinfected containers. *J. Food Prot.* 70: 40-46.

Summary

The possible colonization of the intestines and contamination of broilers after transport to the slaughterhouse with *Campylobacter* strains present in cleaned and disinfected transport containers was investigated. Seven broiler flocks with a *Campylobacter*-free status were sampled once just before loading at the farm and once just before slaughter. On both occasions, samples were also taken from the exterior of the birds and from the intestinal content. Transport containers used to transport the flock were sampled on the farm just before loading the birds. Campylobacters were enumerated and genotyped by Flagellin gene A PCR/restriction fragment length polymorphism and pulsed-field gel electrophoresis. In total, 25 out of the 35 sampled containers were *Campylobacter* contaminated and 30 genotypes were found. Three broiler flocks became colonized on the farm between initial status determination and transport to the slaughterhouse, and three *Campylobacter*-free flocks were externally contaminated after transport. In none of the seven flocks was evidence found of intestinal colonization or co-colonization due to transport in *Campylobacter* contaminated containers.

1. INTRODUCTION

The thermophilic *Campylobacter* species are important causes of human gastroenteritis in most industrialized countries (Tauxe, 1992). Two species, *Campylobacter jejuni* and *Campylobacter coli,* account for approximately 90% of human campylobacteriosis (Vandenberg *et al.*, 2004). The predominant symptoms are acute-to-subacute diarrhea, abdominal pain and fever within 18 h to 8 days after exposure. The diarrhea may be bloody and can be accompanied by nausea and vomiting (Skirrow and Blaser, 2000). A complication that occurs rarely is the autoimmune syndrome Guillain-Barré (Skirrow and Blaser, 2000). Consumption of food of animal origin and poultry products, in particular, has been identified as a major source of infection. Studies have shown that between 20% and 40% of human campylobacteriosis are due to the consumption of chicken meat (Nadeau *et al.*, 2002; Vellinga and Van Loock, 2002).

Chickens become colonized by horizontal transmission, though vertical transmission cannot totally be excluded (Newell and Fearnley, 2003). Once introduced into the broiler house, *Campylobacter* spreads rapidly and all birds become colonized within a few days. The broilers remain colonized until slaughter age (Newell and Fearnley, 2003). The *Campylobacter* carriage rates at flock level range from 3% to more than 90% depending on the region and isolation method applied (Evans and Sayers, 1997; Kapperud *et al.*, 1993). In Belgium, two recent studies reported a *Campylobacter* prevalence at flock level of 67% and 72%, respectively (Herman *et al.*, 2003; Rasschaert *et al.,* 2006). According to Herman *et al.* (2003), there is a discrepancy between the prevalence in flocks at farm level and slaughterhouse level. Five of 11 flocks, which were *Campylobacter* free at farm level, carried campylobacters in the ceca during slaughter one day later. This observation was strengthened by Rasschaert *et al.* (2006), who reported that flocks raised on different farms with no contact during rearing, except that they were slaughtered in the same slaughterhouse on the same day, carried the same strains in the gastrointestinal tract. These two studies indicate the possibility that broilers become colonized during transport from the farm to the slaughterhouse by campylobacters present in the transport crates.

Several studies have already shown the possibility that campylobacters from improperly cleaned and disinfected crates contaminate the surface of the birds transported in these crates (Newell *et al.*, 2001; Slader *et al.*, 2002; Hansson *et al.*, 2005).

The present study aimed to quantify and characterize campylobacters present in transport containers just before loading and on the exterior of the birds before and after transport. Furthermore, it was also determined whether birds can become colonized during transport and resting time by campylobacters present in these transport containers.

2. MATERIALS AND METHODS

Farms. The study was conducted from January 2005 to May 2006. The *Campylobacter* status was determined between two and four days before thinning (=partial depopulation) of the flock or before slaughter of the complete flock. A flock was defined as all birds reared in the same poultry house on a farm. In each poultry house, 60 cecal droppings, (95% CI to detect a prevalence of 5% for more than 10 000 birds) were collected over the entire floor and pooled to result in six samples. When all six samples were *Campylobacter* negative, the flock was assumed to be *Campylobacter* free. Only flocks with a *Campylobacter*-free status were included in the study. Seven flocks from six farms (I to VI) were followed during transport to three slaughterhouses (A, B and G[4]) (Table 1).

Table 1. Overview of the flocks followed during transport.

Flock	Month and year	Farm	Flock	Age of birds	Slaughterhouse
1	January 2005	I	35 000	35 days	A
2	February 2005	II	18 000	35 days	B
3	April 2005	III	20 000	41 days	A
4	May 2005	IV	22 000	42 days[a]	B
5	February 2006	V	25 000	42 days	G
6	March 2006	II	18 000	42 days[a]	B
7	May 2006	VI	35 000	42 days[a]	A

[a]: flocks 4, 6 and 7 were partially depopulated

Sampling on the farm before depopulation. One hour before the (partial) depopulation, six samples of ten cecal droppings each were collected over the entire floor of the poultry house. This was done to verify the *Campylobacter*-free status of the flock. Additionally, 60 birds were randomly picked, and the breast feathers and skin of each bird were sampled with a

[4] Slaughterhouses A and B are the same slaughterhouses as A and B in the previous studies.

swab moistened with peptone water (0.1%). These swabs were pooled to result in six subsamples of ten swabs each.

Per flock, five transport containers (Figure 1, Figure 2) were labeled and sampled before loading the broilers. From each container, four times 400 cm^2 was sampled with four swabs moistened with peptone water (0.1%). These four swabs were pooled to one subsample. Afterwards, the containers were filled with broilers as usual (ca. 300 birds per container, depending on the weight of the broilers), placed on the truck and transported to the slaughterhouse. Time to transport the flock was up to two hours. Afterwards, the broilers were placed outside or beside the hanging area until slaughter. The total transport and resting period before slaughter ranged from 7 to 13 hours.

Sampling at the slaughterhouse. One hour before slaughter, the chickens in the five labeled containers were sampled. From each transport container, ten living birds were randomly picked and sampled. Each bird was held by one technician by the wings, while another technician took swab samples from the crop content, the head (ca. 5 cm^2), breast feathers and skin on the sternum (ca. 25 cm^2) and both feet and toes (ca. 5 cm^2). After the head was sampled, it was pulled forward to stretch the neck to allow a sample to be taken of the crop content. Afterwards, samples were taken from the breast and lastly from the feet. The samples from the crop content and the feet were taken with sterile cotton swabs, whereas the swabs of the breast and the head were taken with sterile cotton balls. All swabs were moistened with peptone water (0.1%) before use. Per container, swabs from the same sampling place (e.g. swabs from the feet from the ten sampled birds from the first container) were pooled to obtain one sample per matrix. After sampling, the bird was put back in the container. When the birds from the five containers were slaughtered, 60 gastrointestinal tracts from the chickens transported in the examined containers were randomly collected just after evisceration.

Description of the transport containers. To transport the broilers, slaughterhouses A and G used plastic drawers placed in metallic frames, so-called loose drawer modules (Figure 1). The drawers containing the broilers were automatically removed from the frames and loaded onto a transport band leading to the hanging area and to the drawer washing facility. The drawers were first soaked in cold water (ca. 15°C) for about 1 to 2 min, then sprayed with cold water and finally sprayed with disinfectant. The frames were sprayed under high pressure with cold water and also sprayed with disinfectant. Finally, the drawers were automatically reloaded into the frames. In slaughterhouse B, metallic auto-dump modules (Figure 2) were

used to transport the birds. Each container was put into the mechanical bird unloading system in which the container moved first to the hanging area, where the container was tilted to unload the birds. Subsequently, the container was moved to the other side of the mechanical bird unloading system where the container was sprayed with water of 55°C and sprayed with disinfectant. The cleaning and disinfection process took ca. 4 min. Finally, the container was removed from the machine and placed on a cleaned truck.

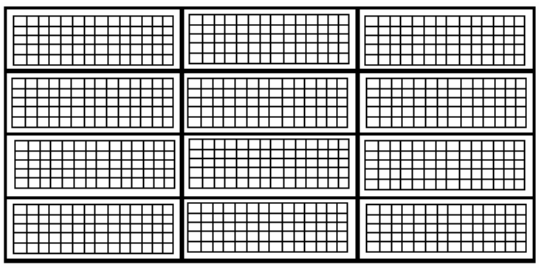

Figure 1. The loose drawer module is the kind of transport container that was used in slaughterhouses A and G. It consists of a metallic frame with 12 plastic drawers.

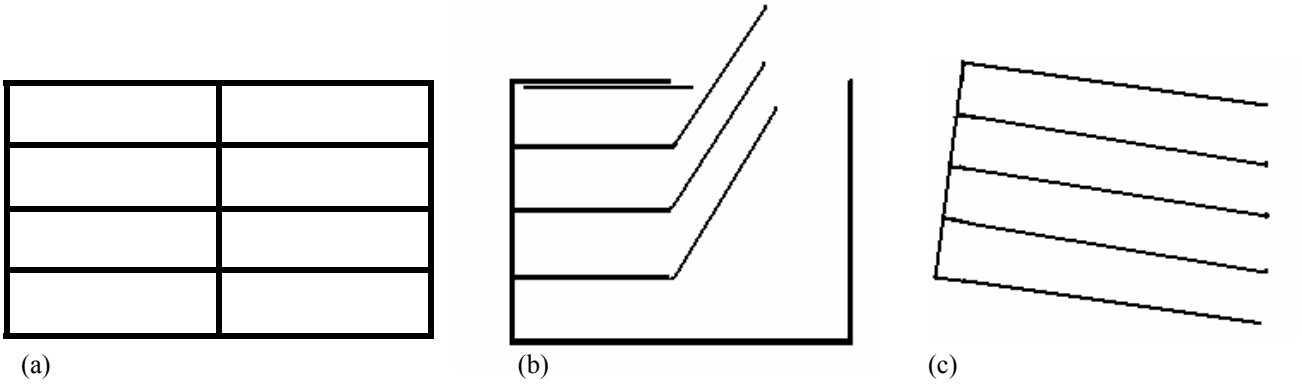

Figure 2. The auto-dump module is the container used by slaughterhouse B. (a) front view, (b) lateral view; the floor of each level is tilted during loading of the birds, (c) lateral view; during unloading, the container is tilted.

In the three slaughterhouses, the trucks were cleaned after each transport. They were swept clean, washed under high pressure with cold water (7 to 16°C) and disinfected with the same product as used to disinfect the containers.

Slaughterhouses A and B used disinfectants based on quaternary ammonium and aldehydes, though slaughterhouse A changed in 2006 to another product formulation but still with the same active ingredients. Slaughterhouse G used a disinfectant with sodium hypochlorite and potassium hydroxide. The concentrations used were according to the manufacturer's recommendations.

Bacteriological culture. Ten gram of the cecal droppings was homogenized by using a stomacher with 90 ml selective Preston broth (nutrient broth n°2 Oxoid CM 67, Basingstoke UK, enriched with 5% (v/v) lysed defibrinated horse blood and 1% Preston supplement (5000 IU polymixin B, 0.010 g rifampicin, 0.0076 g trimethroprim and 0.010 g amphotericin dissolved in 10 ml ethanol)). The swab samples of each container were homogenized in a stomacher laboratory blender with 160 ml Preston broth (1ml=10cm^2). The pooled samples of the head and the pooled samples of the breast were mixed with 100 ml Preston broth and blended in a stomacher, whereas the pooled samples of the crop swabs and the pooled samples of the foot swabs were homogenized in 40 ml Preston broth by vigorously shaking the samples. From each of the 60 gastrointestinal tracts of the broilers, 1 g from the duodenum and 1 g from a cecum were aseptically collected. These samples were pooled to create six duodenum subsamples of 10 g each and six ceca subsamples of 10 each. The pooled samples were homogenized with 90 ml of Preston broth in a stomacher blender.

After homogenization, 100 µl of all homogenates was inoculated by the quantitative spiral plating method on two modified cefoperazone charcoal deoxycholate agar plates (mCCDA, Oxoid CM 739 plus SR155, Basingstoke, UK). All plates and homogenates were then incubated at 42°C under microaerobic conditions (6% CO_2, 6% H_2, 4% O_2 and 84% N_2). After an incubation of 24 h and 48 h, respectively, 10 µl of each enrichment broth was plated onto an mCCDA plate. Typical colonies were examined by Gram-staining, subcultured and stored in whole horse blood at –80°C for further examination. As suggested in the study of Newell *et al.* (2001), campylobacters obtained by direct plating and following enrichment of the same sample were collected and further characterized to increase the number of isolates and different genotypes.

Species identification and characterization of *Campylobacter* isolates. All isolates were identified at species level according to the PCR assay of Vandamme *et al.* (1997). Isolates were further characterized at strain level by a combination of flagellin gene A PCR/restriction fragment length polymorphism (*fla*A typing) and pulsed-field gel electrophoresis. *Fla*A typing was applied according the method of Nachamkin *et al.* (1993) with *Dde*I (Promega, Madison, Wisconsin, USA) as the restriction enzyme. Pulsed-field gel electrophoresis was performed as described by Rasschaert *et al.* (2006). In brief, isolates were grown on mCCDA for 18 h at 42°C under microaerobic conditions. The method of Olsen *et al.* (1994) was followed to prepare the plugs. Plug slices were digested for 18 h with 40 U of *Sma*I. DNA

fragments were separated by Chefmapper in a 1% Seakem agarose (Biowhittaker Molecular Applications, Rockland, Maine). The running conditions were 6 V/cm at 14°C in 0.5 x Tris-borate-EDTA buffer for 22 h with a ramping time from 4 to 40 s. All electophoretic gel images were analyzed by GelCompar 3.0 (Applied Maths, Sint-Martens-Latem, Belgium). The similarities among fingerprints were calculated using the Dice-coefficient (optimization and position tolerance of 1%), and an average linkage (UPGMA, unweighted-pair group method using arithmetic averages) was derived from the fingerprints. A delineation level of 90% (Rasschaert *et al.*, 2006) was used to discriminate different genotypes. The genotypes were designated numerically on the basis of the profile patterns obtained by *fla*A and PFGE (i.e. genotype 1.1 had profile number 1 for *fla*A and profile number 1 for PFGE).

3. RESULTS

Table 2. Campylobacters in the cleaned and disinfected transport containers just before loading the birds (CFU/cm^2)

	Container 1	**Container 2**	**Container 3**	**Container 4**	**Container 5**
Flock 1[a]	2	90	5	3	P
Flock 2	P	A	P	1	P
Flock 3[a]	82	12	2	14	27
Flock 4	5	NC	P	1	A
Flock 5	P	NC	1	P	P
Flock 6	A	A	A	A	A
Flock 7	A	A	P	A	P

[a]: these crates were used on Sunday and were thus cleaned on Friday.
P: present, campylobacters were recovered after enrichment
A: absent, no campylobacters were recovered after enrichment
NC: not countable (swarming of the campylobacters)

Table 2 gives an overview of the presence of *Campylobacter* in the cleaned and disinfected containers before loading the birds. In total, 25 containers (71%) were contaminated with campylobacters: 15 containers yielded *Campylobacter* by direct plating whereas ten containers were positive following enrichment only. In total, 117 *Campylobacter* isolates from the containers were further examined. Sixty-three isolates were identified as *C. jejuni* (54%), whereas the others were identified as *C. coli* (46%). In total, 30 genotypes were isolated. Individual transport containers were contaminated with up to four genotypes, whereas the five examined containers used to transport the flock were contaminated with up to eight genotypes (Table 3).

The intention of the study was to include only flocks with an initial *Campylobacter*-free status. However, three of the seven studied flocks became colonized before depopulation, though no campylobacters were detected at initial status determination, which was two to four days before transport. As shown in Table 4, the cecal droppings of flocks 3, 4 and 7 were contaminated with *C. jejuni* genotype 14.14, *C. coli* genotype 12.24 and *C. jejuni* genotype 32.32, respectively. The exteriors of the chickens of these three flocks were contaminated after transport. Since the breasts of the chickens were already contaminated before transport with the same genotype as isolated from the cecal droppings, no colonies of the surface of the broilers after transport were picked for further characterization. Colonies were picked from the crop and intestines to investigate the possibility of colonization by other *Campylobacter* strains present in the transport container. In all cases, the isolates picked were of the same genotype as the isolates from the cecal droppings collected at the farm (Table 4). For flocks 4 and 7, campylobacters on the exterior of the birds and in the gastrointestinal tract were enumerated as shown in Table 5.

The other four flocks (flocks 1, 2, 5 and 6) maintained the *Campylobacter*-free status in the period between status determination and transport. In none of these flocks were campylobacters isolated from the gastrointestinal tract after transport (Table 4). Although the flocks were *Campylobacter* free before transport, only one flock (flock 5) was not found to be externally contaminated after transport. For flock 1 as well as flock 2, the birds transported in two of the sampled containers were contaminated with two strains on the head, breast, or both. For flock 6, the broilers of only one transport container were contaminated on the head and the breast, each with a different strain (Table 4). On all occasions, these campylobacters were found following enrichment only; hence, fewer than 20 CFU/cm^2 were present on the head and fewer than 4 CFU/cm^2 were present on the breast. The genotypes found on the exterior of the birds did not always correspond with the genotypes found in the transport container in which they were transported: genotypes 2.2 and 13.13 were found in one of the other four sampled containers, whereas genotypes 9.9, 30.30 and 31.31 were found in none of the sampled containers used to transport the flock. Though genotypes 30.30 and 31.31 were found on the exterior of the chickens of flock 6, no campylobacters were detected in the five sampled containers.

Table 3. Species and genotypes of the isolated campylobacters from the cleaned and disinfected transport containers

	Container 1				Container 2				Container 3				Container 4				Container 5			
	direct		enriched		direct		enriched		direct		enriched		direct		enriched		direct		enriched	
Flock 1	*C. jejuni*	1.1[a]	*C. jejuni*	2.2'[b]	*C. coli*	3.3	*C. coli*	3.3	*C. coli*	3.3	*C. jejuni*	5.5	*C. jejuni*	5.5	*C. jejuni*	6.6			*C. jejuni*	5.5
					C. jejuni	3.4			*C. jejuni*	3.4					*C. jejuni*	2.2			*C. jejuni*	7.7
																			C. jejuni	8.8
Flock 2			*C. jejuni*	5.10							*C. coli*	11.12	*C. jejuni*	5.10	*C. jejuni*	5.10			*C. jejuni*	9.15
											C. coli	12.12			*C. coli*	12.12			*C. coli*	13.13
Flock 3	*C. coli*	12.15	*C. coli*	20.20	*C. coli*	16.16	*C. coli*	7.17	*C. coli*	12.15	*C. coli*	12.15	*C. coli*	19.19	*C. coli*	20.20	*C. coli*	12.15	*C. coli*	7.17
	C. coli	16.16			*C. coli*	7.17			*C. coli*	21.21	*C. coli*	7.17					*C. coli*	21.21	*C. coli*	21.21
	C. coli	7.17			*C. jejuni*	18.18														
Flock 4	*C. jejuni*	22.14	*C. jejuni*	5.25	*C. coli*	23.23'	*C. coli*	23.23			*C. coli*	23.23	*C. coli*	23.23	*C. coli*	23.23				
	C. coli	26.26											*C. coli*	26.26						
Flock 5			*C. jejuni*	6.27	*C. jejuni*	6.27	*C. jejuni*	6.27			*C. jejuni*	6.27			*C. jejuni*	6.27			*C. jejuni*	6.27
											C. jejuni	29.29			*C. jejuni*	5.28				
Flock 6																				
Flock 7											*C. coli*	34.34							*C. jejuni*	33.33

[a]: genotypes were designated numerically based on profile patterns obtained by *fla*A (first number) and PFGE (second number) (i.e. genotype 1.1 had profile number 1 for *fla*A and profile number 1 for PFGE).
[b]: an apostrophe means that the PFGE profile is considered closely related to the strain without apostrophe according to the criteria of Tenover *et al.* (1995)

Table 4. Surface contamination and intestinal colonization on the farm and after transport

	FARM				SLAUGHTERHOUSE							
	cecal droppings		**surface of chickens (breast)**		**surface of chickens**			**crop**		**ceca**		**duodenum**	
Flock 1					breast (Cont 4)[a]	*C. jejuni*	2.2 [b]						
					head (Cont 4)	*C. jejuni*	9.9						
					breast (Cont 5)	*C. jejuni*	2.2						
Flock 2					breast (Cont 3)	*C. coli*	13.13						
					head (Cont 4)	*C. jejuni*	5.10						
Flock 3	*C. jejuni*	14.14	*C. jejuni*	14.14	NT			*C. jejuni*	14.14	*C. jejuni*	14.14	*C. jejuni*	14.14
Flock 4	*C. coli*	12.24	*C. coli*	12.24	NT			*C.coli*	12.24	*C. coli*	12.24	*C. coli*	12.24
Flock 5													
Flock 6					breast (Cont 3)	*C. jejuni*	30.30						
					head (Cont 3)	*C. coli*	31.31						
Flock 7	*C. jejuni*	32.32	*C. jejuni*	32.32	NT			*C. jejuni*	32.32	*C. jejuni*	32.32	*C. jejuni*	32.32

[a]: in the slaughterhouses, birds were sampled from five containers. The container in which the birds were externally *Campylobacter* contaminated is indicated in parentheses.
[b]: genotypes were designated numerically based on profile patterns obtained by *fla*A (first number) and PFGE (second number) (i.e. genotype 1.1 had profile number 1 for *fla*A and profile number 1 for PFGE).
NT: not tested

Table 5. Mean counts for *Campylobacter* from the exterior of the chickens just before slaughter and of the gastrointestinal tract for flocks 4 and 7 (in $\log_{10}$ CFU)

	Flock 4	Flock 7
Breast	2.18/cm^2	3.52/cm^2
Head	3.22/cm^2	4.11/cm^2
Feet	4.72/cm^2	3.98/cm^2
Crop	4.22/crop	3.48/crop
Duodenum	3.80/g	6.32/g
Ceca	> 6.88/g	> 6.88/g

4. DISCUSSION

Crate washing to remove campylobacters is recognized as being largely ineffective (Berndtson *et al.*, 1996a; Slader *et al.*, 2002; Ramabu *et al.*, 2004). To our knowledge, this is the first study in which campylobacters in the containers were quantified and genotyped. Twenty-five containers were *Campylobacter* contaminated, and multiple genotypes were present in these containers. All containers of slaughterhouse A were contaminated on the first two sampling occasions, whereas on the third sampling occasion, only two containers were found positive. Since the second sampling, this slaughterhouse paid more attention to the cleaning and disinfection process in general and also started to use another disinfection product, though with the same active ingredients. In the present study, the containers were sampled just before loading the birds at the farm, whereas in other studies the containers were sampled just after washing (Slader *et al.*, 2002; Hansson *et al.*, 2005). The time between the cleaning and disinfection process and the reuse of the transport containers may range from hours to days. In the present study, all containers were cleaned and disinfected at least 12 hours before sampling and loading the birds. The containers in which flock 1 and 3 were transported were even washed and disinfected at least 60 hours before sampling. Since *Campylobacter* is sensitive to dryness, it is important to detect the presence of campylobacters in the containers just before loading the birds. The number of campylobacters can diminish, or certain genotypes can die off in the time between container washing and loading the birds. Although visually dry, some containers contained a substantial amount of campylobacters of different genotypes, even though *Campylobacter* is sensitive to dryness. In a study of Berrang *et al.* (2001), naturally *Campylobacter* contaminated containers that were allowed to dry for 48 hours without a cleaning and disinfection process yielded fewer campylobacters (only after enrichment or even none) than the containers in the present study which were washed and

disinfected and had time to dry for more than 60 hours (flock 1 and 3). It is also quite remarkable that nearly half of the isolates from the transport containers were *C. coli.* This may indicate that *C. coli* is less sensitive to dryness.

None of the *Campylobacter* flocks were colonized after transport with strains found in the transport containers. This contrasts with the results of Jacobs-Reitsma and Bolder (1998), who kept *Campylobacter*-free flocks for 4 hours in naturally contaminated transport containers. However, the campylobacters in that experiment were freshly excreted, whereas in the present study, the containers were disinfected and the organic residue had time to dry. In the study of Hansson *et al.* (2005), campylobacters were isolated from the cloaca after transport from five flocks of 26 flocks with an initial *Campylobacter*-free status. However, *Campylobacter* contamination of the cloaca is probably not a good indicator for intestinal colonization. The cloaca can become contaminated by contact with the contaminated containers, without colonization of the intestines. Furthermore, cloacal swabs are subjected to cross-contamination from the skin (Sandberg *et al.*, 2006).

Regarding the flocks which maintained the *Campylobacter*-free status, 25% of the broilers were contaminated on the head, breast, or both after transport. Although this contamination is restricted to a limited number of birds, it can be important, since these birds can cause cross-contamination during the slaughter process. In the present study, the genotypes from the transport containers and from the surface of the birds did not always correspond. Three out of the six genotypes isolated from the exterior of the chickens, were not isolated from any of the five crates used to transport the birds. As the birds were not contaminated externally just before loading, and as they were sampled at the slaughterhouse while they were still in the crates, they must have been contaminated during loading by the capture team, or more likely, the crates harbored more genotypes than detected. Therefore, in further studies, it is advisable to swab more than 1600 cm^2 per container. In the study of Hansson *et al.* (2005), 10 from the 26 *Campylobacter*-free flocks transported in *Campylobacter* contaminated crates had contaminated neck skin samples at slaughter. It is possible that most neck skins became contaminated by the slaughter environment and not by the containers as the strains isolated from the neck skins did not correspond to those found in the containers.

From the three flocks that became *Campylobacter* colonized just before slaughter, two flocks were partially depopulated. It is possible that thinning of the flock brought *Campylobacter* into the broiler house, since partial depopulation is known to be a risk factor (Hald *et al.*,

2000). Flock 7 was partially depopulated at the age of 39 days. The next day, the *Campylobacter* status was determined and found to be *Campylobacter* free. However, the cecal droppings collected when the birds were 42 days old, appeared to be *Campylobacter* positive. Once campylobacters are introduced into the broiler house, it is known that birds become colonized within a few days (Corry and Atabay, 2001; Newell and Fearnley, 2003).

According to several studies (Oosterom *et al.*, 1983; Izat *et al.,* 1988; Berrang *et al.*, 2000), carcass contamination during slaughter can occur during scalding, plucking and evisceration, when the gastrointestinal tract leaks possible *Campylobacter* contaminated fecal material. A second way of contamination of the carcasses is by cross-contamination of previously slaughtered flocks or via the slaughter equipment (Rasschaert *et al.*, 2006). The present study reveals that transport containers, even after the cleaning and disinfection process and with a drying time of at least 12 hours, can still harbor a lot of different *Campylobacter* genotypes. After the transport of flocks in such containers, no intestinal colonization of the flocks by campylobacters present in the transport containers was observed, though this transport can lead to surface *Campylobacter* contamination of the broilers. This superficial contamination also contributes to the level found on the fully processed carcasses (Stern *et al.*, 1995; Berrang *et al.*, 2001).

GENERAL DISCUSSION

1. Introduction

Bacterial foodborne infections are a common cause of human gastrointestinal disease in the industrialized world. *Salmonella* and *Campylobacter* account for over 90% of all reported cases of bacterial food poisoning. Case-control studies and epidemiological studies in which strains isolated from various sources were compared with those causing infections in humans, have provided evidence that poultry meat represents an important risk factor. Attempts to sensibilize the public on the proper handling and preparation of poultry meat have been made, but have not been of much influence. Another approach to decrease the risk for human infection is to avoid that poultry meat contaminated with *Salmonella* or *Campylobacter*, or both, reach the consumer. This can be achieved by reduction of the number of colonized broiler flocks at the farm by vaccinating breeder flocks, improved hygiene measures, and treatment of water and feed. As important is to control the transport and the slaughter process to avoid contamination of the end product by spilling of the gastrointestinal content or by cross-contamination. Nevertheless, it has been reported that carcasses were contaminated after processing, even when the flocks were *Salmonella* or *Campylobacter* free at the farm. The aim of this PhD work was to study the contamination of poultry with *Salmonella* and *Campylobacter* caused by transport in contaminated containers, by gastrointestinal spillage of colonized flocks, and by cross-contamination from previously slaughtered flocks or the slaughter environment. The study was performed by means of molecular tools which are evaluated in the following section.

2. Evaluation of the molecular techniques used to discriminate *Salmonella* and *Campylobacter* isolates

The internationally used method for subtyping *Salmonella* isolates is serotyping according to the Kauffmann-White scheme (Popoff and Le Minor, 1997). Serotyping is expensive, time-consuming, variable and often restricted to national reference laboratories. In contrast, PCR-based methods are easy and rapid to perform, relatively cheap, and available in a lot of laboratories. At the start of this PhD work, there was no consensus about a molecular technique to correlate *Salmonella* serotypes with genotypes. As a large number of *Salmonella* isolates was planned to be serotyped, it was first investigated if rep-PCR could be used as a good alternative to discriminate *Salmonella* isolates at the serotype level. Based on the results

presented in Chapter I, it was concluded that rep-PCR with the ERIC primer set or the $(GTG)_5$ primer cluster *Salmonella* isolates of the same serotype together and that, consequently, only one or a few isolates per cluster have to be serotyped. Furthermore, rep-PCR is able to produce fingerprints for strains which are not typeable by serotyping. Finally, the combination of rep-PCR and serotyping may reveal serotyping errors or laboratory errors made during rep-PCR. A disadvantage of this technique is the low reproducibility, meaning that profiles obtained by different PCR runs cannot be compared unless a representative of each cluster is included in each new PCR run. Therefore, rep-PCR is only cost-efficient in studies in which a large number of isolates of a limited number of serotypes is expected. This also means that a library with fingerprints of serotypes cannot be created, and that profiles cannot be compared between different laboratories as can be done with for example PFGE. Since the publication of this study, nearly 1500 *Salmonella* isolates were successfully clustered by ERIC-PCR. Fourteen percent of these isolates were serotyped, which resulted in a reduction of more than 50% in the total cost compared to serotyping all isolates.

In the present work, *fla*A typing was used as a first discriminatory method for characterizing *Campylobacter* isolates. Approximately 600 *Campylobacter* isolates were characterized by *fla*A typing and all generated fingerprints with the exception of the *C. lari* isolates. As recommended by Campynet (Harrington *et al.*, 2003), *fla*A typing was supported by a second molecular tool. PFGE was chosen due to its high discriminatory potential and high reproducibility. The results presented in Chapter III demonstrated that these two techniques are complementary. As expected, PFGE was able to further discriminate certain *fla*A types. On the other hand, *fla*A typing was in some cases able to subtype certain PFGE types. Furthermore, the combination of these two techniques allowed the identification of closely related strains. This highlights the importance of using a combination of two molecular methods. Nevertheless, in some epidemiological studies only one technique has been used to characterize *Campylobacter* isolates (Rivoal *et al.*, 1999; Hansson *et al.*, 2005). The results of these studies should be interpreted with care. For epidemiological studies the number of samples to be taken or the number of isolates per sample to be characterized are also important and can influence the obtained results. However, according to Newell *et al.* (2001), flocks are colonized by maximal three *Campylobacter* genotypes. This is also confirmed by studies in which extensive sampling methods were compared to smaller ones (Newell *et al.* 2001). Furthermore, enrichment is considered the optimal method for recovery of small numbers of campylobacters or campylobacters under stress conditions. Therefore, Newell *et*

al. (2001) recommend to use enrichment for isolation of campylobacters of poultry carcasses. However, enrichment can select certain strains although it is reported that this selection is not so obvious in cultures from cecal contents (Newell *et al.* 2001) .

3. Prevalence of flock colonization at slaughterhouse level

The prevalence of flocks colonized with *Salmonella* and *Campylobacter* found in the present work is within the range of other reported prevalences: from 0% in Sweden to 77% in Canada for *Salmonella*, and from 3% in Finland to more than 90% in the UK for *Campylobacter* (Renwick *et al.*, 1992; Wierup *et al.*, 1995; Evans and Sayers, 2000; Perko-Mäkelä *et al.*, 2002). With the exception of the Nordic countries where a lower *Salmonella* and *Campylobacter* prevalence occurs, it is difficult to compare the prevalence between countries due to different sampling and isolation methods. The age of the birds at the moment of sampling and the type of sample are important factors. For example, in a Belgian study in which 18 broiler flocks were followed from hatching to the slaughterhouse, ten flocks received a *Salmonella*-positive status during rearing, but the number of positive flocks dropped to six at slaughter age (Heyndrickx *et al.*, 2002). Furthermore, birds can change from *Salmonella* shedders to *Salmonella* carriers, or *vice versa*. By collecting only fecal material, carrier flocks can be wrongly identified as *Salmonella* free. The prevalence of flocks colonized with *Campylobacter* increases with age (Evans and Sayers, 1997; van de Giessen *et al.*, 2006). This was also observed in the study presented in Chapter VI: three of the seven followed broiler flocks became *Campylobacter* colonized between two and four days before slaughter. For these reasons, it is important to take samples as near as possible to slaughter.

It has been observed that flocks can become colonized with *Salmonella* during transport (Rigby and Pettit, 1980). There are also some indications that flocks can also become contaminated with *Campylobacter* during transport. In the study of Herman *et al.* (2002) for example, it was demonstrated that flocks with a *Campylobacter*-negative status at the end of the rearing period at the farm, were colonized at the moment of slaughter. In the present work (Chapter III), it was observed that -on some occasions- flocks slaughtered on the same day in the same slaughterhouse were colonized with the same *Campylobacter* strains, even though there had not been any contact between them during rearing. Flocks processed in different slaughterhouses or on different days were seldom colonized with the same strains. *Campylobacter* strains isolated from the ceca were often different from those present in the

crop. The strains in the upper gastrointestinal tract may have originated from a recent infection during transport in improperly cleaned and disinfected containers. In several studies, it was demonstrated that the containers used in practice were still contaminated with *Campylobacter*. However, in most studies the number of samples was limited, the campylobacters were not quantified and characterized, and the containers were sampled just after the cleaning and disinfection process (Newell *et al.*, 2001; Slader *et al.,* 2002; Hansson *et al.*, 2005). As *Campylobacter* is known to be sensitive to dryness, the number of campylobacters present in the transport containers can decrease during the period between the cleaning and disinfection process and actually using the containers. Therefore, in the study described in Chapter VI, it was chosen to sample the containers on the farm just before loading the birds. The study showed that the majority of the examined containers were still contaminated with campylobacters. Nevertheless, none of the sampled flocks became colonized with strains isolated from the containers. But external contamination of birds from some flocks with strains isolated from the containers was observed. Although this contamination was limited, this may be important when flocks which were *Campylobacter* free at the farm, are transported and processed.

In some studies, cloacal samples are taken in the slaughterhouse to examine flock colonization (Hansson *et al.*, 2005; Lindmark *et al.*, 2006). Although this sampling method is easy and fast, this is in our opinion not a good sampling method. Contamination of the cloaca by contact with for example contaminated containers during transport without colonization of the intestines cannot be excluded. As the ceca are the primary sites of colonization for *Salmonella* as well as for *Campylobacter* (Fanelli *et al.*, 1970; Beery *et al.*, 1988; Achen *et al.*, 1998), they seem the most obvious sampling site. However, a research group has demonstrated that preslaughter feed withdrawal results in an increased incidence of *Salmonella*- and *Campylobacter*-positive crops and that this number may even exceed the number of positive ceca (Hargis *et al.*, 1995; Ramirez *et al.*, 1997; Byrd *et al.*, 1998; Corrier *et al.*, 1999). As these studies have been performed on individual birds, it was investigated if these results can be extrapolated to the flock level, and consequently, if sampling the crops of the birds is better to determine flock colonization (Chapter IV). It was demonstrated for flocks being processed that the duodenum was most often colonized with *Salmonella* or *Campylobacter*, followed by the ceca and finally the crop. Sampling only the duodenum was sufficient to determine the *Campylobacter* status. For identification of all flocks colonized with *Salmonella* all three sites had to be sampled. However, it should be kept in mind that for

epidemiological studies, sampling different sites of the gastrointestinal tract is necessary as the genotypes of the isolates can be different.

In execution of regulation EC/2160/2003 on the control of *Salmonella* and other specified foodborne zoonotic agents, the E.U. has published a study about the *Salmonella* prevalence in laying hen flocks (EFSA, 2006a). The reported prevalence for Belgium (35%) was almost half of the prevalence found in this PhD work. In the EFSA study, the prevalence was determined by collecting fecal and environmental samples during the last nine weeks of the production period of the flocks. As explained above, the birds can acquire a new infection after status determination or can change from *Salmonella* carriers to shedders, or *vice versa*. Another explanation for this discrepancy is that in the time span between sampling the laying hen flocks for the present work (beginning of 2003) and sampling the laying hen flocks for the EFSA study (end of 2004-beginning 2005) the situation has ameliorated due to an increased vaccination of laying hen flocks.

It is generally accepted that flocks -once colonized with *Campylobacter*- remain colonized until the slaughter age of six weeks. From the age of eight weeks, the number of infected birds and the number of campylobacters may gradually decrease (Achen *et al.*, 1998; Newell and Wagenaar, 2000). However, instead of reduced colonization, it was demonstrated in Chapter IV that colonization in laying hen and breeder flocks is higher than in broiler flocks, and this in terms of the number of flocks colonized, as well as the number of species isolated from the gastrointestinal tract. This requires further investigation.

4. Carcass contamination during poultry processing

One of the aims of this PhD work was to study, for both bacteria, the contribution of gastrointestinal colonization and cross-contamination to carcass contamination during poultry slaughter (Chapter II and III). In several studies, contamination of carcasses during processing of poultry flocks has been investigated. In these studies, however, samples were taken from only one, or at the most, two successively slaughtered flocks (Rivoal *et al.*, 1999; Newell *et al.*, 2001; Corry *et al.*, 2002; Slader *et al.*, 2002; Olsen *et al.*, 2003). In the present work, it was chosen to sample several successively slaughtered flocks to determine the contamination level caused by the gastrointestinal content of the flock itself or by cross-contamination.

Although the same samples from the same flocks were processed for the presence of *Salmonella* and *Campylobacter*, the results for both pathogens were quite different.

Although only a small proportion of the broiler flocks (13%) arriving at the slaughterhouse were colonized with *Salmonella*, carcasses of more than half of the broiler flocks (55%) were contaminated after slaughter. This is of concern because a lot of efforts are made to eradicate *Salmonella* such as vaccination of the breeder flocks, improved hygiene measures at the farm, and implementation of logistic slaughter to reduce cross-contamination. The origin of the majority of the strains isolated from the carcasses was unknown since they were not isolated from the gastrointestinal content of any of the flocks slaughtered that day. *Salmonella* cross-contamination was a major problem in different slaughterhouses, regardless whether the strains originated from the gastrointestinal tract, or had an unknown origin. Some strains were able to contaminate all flocks slaughtered on the same sampling day. Based on these observations, it was hypothesized that the slaughter equipment can be contaminated with *Salmonella* before the onset of slaughter, and consequently, act as a primary source of carcass contamination. This hypothesis was confirmed by the study presented in chapter V. The slaughter line was contaminated with *Salmonella* strains before the onset of slaughter in two of the three examined slaughterhouses. During slaughter, carcasses of the first flock became contaminated with the same strains as isolated from the slaughter line. Logistic slaughter is a control measure implemented in Belgium to maintain *Salmonella*-free flocks *Salmonella* free during processing. However, one of the prerequisites of logistic slaughter is that the slaughter line is completely *Salmonella* free before the onset of slaughter. The only slaughterhouse that achieved this goal, was visually cleaner than the other two. It was the only slaughterhouse in which the daily cleaning and disinfection process was subcontracted to an external firm. In the other two slaughterhouses, personnel of the slaughterhouse was responsible for the cleaning and disinfection process. It might be that an external firm is more rigorous in cleaning and disinfection of the slaughter environment. This hypothesis –based on only one observation- certainly requires further investigation.

Compared to the high percentage of flocks colonized with *Campylobacter* arriving at the slaughterhouse (72%), only a small increase in flocks contaminated with *Campylobacter* after processing (79%) was observed. In most cases, the strains isolated from the carcasses of a flock were the same as those found in the gastrointestinal tract from the same flock or, in some cases, from one or two previously slaughtered flocks. Only once, carcasses of a flock

slaughtered first were contaminated with a *Campylobacter* strain of which the origin was unclear. A possible explanation may be that this strain originated from the slaughter equipment and, consequently, had survived the cleaning and disinfection process. After the publication of this study (Chapter III), a Swedish study with a similar set-up was published (Lindmark *et al.*, 2006). Although the Swedish prevalence for flock colonization is much lower than the Belgian (Hansson *et al.*, 2004), the same conclusions were drawn about the contamination of carcasses during processing of poultry flocks. The strains present on carcasses were, in most cases, also isolated from the cloacal samples of the same flock, or in some cases, from the cloacal samples of the preceding flock. Only once, a strain isolated from carcasses was the same as the one isolated from a flock slaughtered the previous day.

5. Conclusions, recommendations and future perspectives

The contribution of the gastrointestinal colonization to carcass contamination with *Campylobacter* was demonstrated to be high. It is almost unavoidable that carcasses of a colonized flock become contaminated during processing for two reasons. Firstly, the within flock prevalence is often 100% and birds harbor a high number of campylobacters in the intestines, except for recent infections. Consequently, the exterior of colonized flocks are already contaminated with *Campylobacter* at the farm, and this contamination is further increased during transport (Stern *et al.*, 1995). Therefore, it should be noted that the contamination on the exteriors of the birds can already be high before processing. Secondly, as the gastrointestinal tract of colonized flocks harbor a high number of campylobacters, even a small amount of gastrointestinal content that is spilled on the carcass during processing can cause a significant increase in the number of campylobacters on the carcass. The best options to reduce carcass contamination are either decontamination of the final product or reduction of the number of flocks colonized with *Campylobacter*. Chemical decontamination and irradiation are forbidden in the E.U., and other decontamination methods are expensive, still in development, or cause changes in the appearance of the product. According to many studies, the most important routes for flock colonization are related to biosecurity. However, the implementation of improved hygiene measures does not seem to contribute in practice to the reduction of flock prevalence. Therefore, more research should be conducted to the identification of the source of colonization or the prevention of flock colonization. In addition,

partial flock depopulation, as applied frequently, should be evaluated as an important source of introducing *Campylobacter* in Belgian poultry flocks.

In this PhD work, it was demonstrated that the prevalence of *Salmonella* colonized broiler flocks was low. This is due to the major efforts done at farm level such as vaccination of the breeder flocks. As less *Salmonella* colonized flocks enter the slaughter line, the importance of contamination by the slaughter environment increases as demonstrated in this work. In execution of the Commission Regulation No. 2073/2005 on 'microbiological criteria for foodstuffs', neck skins have to be sampled in poultry slaughterhouses at regular time points to examine possible contamination of carcasses with *Salmonella*. In the case that carcasses of flocks with a *Salmonella*-free status are contaminated with *Salmonella* following slaughter, one of the options that should be considered is cross-contamination from the slaughter line or containers. The slaughterhouse should evaluate the used cleaning and disinfection process for both, and if necessary, investigate how this process can be improved. Once implemented, the effectiveness of the cleaning and disinfection process should be monitored at regular time intervals. However, it is difficult to remove all organic material from transport containers, especially dried material, with the crate washing equipment used today consisting of a combination of only water, pressure, disinfectants and, in some cases, temperature. Therefore, development of equipment to clean and disinfect crates, for example equipped with brushes in order to apply more mechanical forces during the cleaning process, should also further be investigated.

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SUMMARY

Salmonella and *Campylobacter* are two important bacterial causes of gastroenteritis in the industrialized world. In Belgium, 48 cases of salmonellosis and 66 cases of campylobacteriosis per 100 000 habitants were reported in 2005. Handling or consumption of contaminated poultry meat is considered an important source of the transmission for both pathogens to humans. Most studies focus on the farm level to reduce the number of flocks colonized with these bacteria in order to lower the risk for human infection. However, it has been reported that, after processing, carcasses have been contaminated even when the flocks were *Salmonella* or *Campylobacter* free at the farm. Therefore, the general aim of this PhD work was to study *Salmonella* and *Campylobacter* contamination of poultry during transport and slaughter by means of molecular tools.

In the literature study, an overview is presented of the taxonomy and the characteristics of *Salmonella* and *Campylobacter*. In addition, the clinical aspects, the epidemiology and the possible sources of human infection caused by these two bacteria are discussed. Furthermore, flock prevalence and possible transmission routes for flock colonization, transport to the slaughterhouse, the Belgian slaughter process and contamination of carcasses during processing are discussed. Finally, the most commonly used molecular techniques for characterization of *Salmonella* and *Campylobacter* isolates are described.

In Chapter I, an evaluation of five repetitive-sequence-based PCR typing methods for the discrimination of *Salmonella enterica* isolates at serotype level is presented. Based on the number, even distribution over the whole fingerprints, and clarity of bands in the fingerprints, the ERIC primer set and the $(GTG)_5$ primer were chosen for use in further experiments. Rreproducibility was evaluated on different lysates of five *Salmonella* serotypes using different PCR runs in the same thermal cycler. Reproducibility was poor between different PCR runs, but high within the same PCR run. Additionally, 80 different serotypes and five isolates which were not typeable by serotyping were fingerprinted. All strains were typeable and generated unique fingerprints, except for some strains with incomplete antigenic codes. Finally, strains of the same serotype, but genetically different, were fingerprinted to examine the genetic diversity within serotypes. A serotype did not always correlate to only one ERIC or $(GTG)_5$ fingerprint but the fingerprint heterogeneity within a serotype was limited. Since the reproducibility of isolates in one PCR run, the discriminatory power, and the genetic diversity of the fingerprint were similar for the ERIC primer set and the $(GTG)_5$ primer, both primer sets are equally useful to discriminate *Salmonella* serotypes. In the studies presented in

Chapters II, IV and V, ERIC-PCR was used to limit the number of *Salmonella* isolates to be serotyped.

Chapter II describes the contribution of gastrointestinal colonization and cross-contamination to *Salmonella* carcass contamination during poultry slaughter. Although only 13% of the broiler flocks were colonized with *Salmonella* in the gastrointestinal tract at the time of slaughter, carcasses of 55% of the broiler flocks were contaminated after slaughter. Concerning the laying hen and breeder flocks, 69% of the flocks were colonized in the gastrointestinal tract, and carcasses of all flocks were contaminated after slaughter. Characterization of the isolates showed that the majority of the strains isolated from the carcasses did not originate from the gastrointestinal content of any of the flocks slaughtered that sampling day. *Salmonella* cross-contamination was a major problem in different slaughterhouses, regardless if the isolated strains originated from the gastrointestinal tract or were of unknown origin. Certain strains were isolated from all flocks slaughtered on one sampling day. Cross-contamination was not only observed to following slaughtered flocks but also to preceding flocks.

In Chapter III, the contribution of gastrointestinal colonization and cross-contamination to carcass contamination with *Campylobacter* during poultry slaughter is described. Seventy-two percent of the broiler flocks arriving at the slaughterhouse were colonized with campylobacters. After slaughter, 79% of the flocks yielded contaminated carcasses. Isolates were identified as *Campylobacter jejuni* (89%), *Campylobacter coli* (8.7%) and *Campylobacter lari* (2.3%). The combination of two molecular methods allowed the discrimination of 27 genotypes. Overall, genotypes of isolates from the carcasses of a flock were the same as those found in the gastrointestinal tract from the same flock. From carcasses of only six flocks (15%), strains were isolated which were also found in the gastrointestinal tract from one or two flocks slaughtered before. Although cross-contamination was limited, four of these six flocks were initially *Campylobacter* free and might not have had contaminated carcasses after logistic slaughtering.

In Chapter IV, the results of a study with two different objectives are presented. The first objective was to investigate the association between the concurrent colonization with *Salmonella* and *Campylobacter*. The second objective was to determine the best sampling site for status determination at the slaughterhouse. Samples were taken from three different sites of the gastrointestinal tract, namely from the crop, the duodenum and the ceca. No association

was found between *Salmonella* and *Campylobacter* prevalence within broiler flocks. Since all laying hen and breeder flocks were *Campylobacter* colonized, no association between the concurrent colonization of flocks with these two bacteria could be determined. The duodenum was demonstrated to be most often colonized with the two bacteria, followed by the ceca and finally the crop. Sampling only the duodenum was sufficient to determine the *Campylobacter* status, whereas for identification of all *Salmonella* colonized flocks all three sites needed to be sampled.

The aim of the study, described in Chapter V, was to assess the impact of *Salmonella* present on the slaughter line on broiler carcass contamination. In two of the three sampled slaughterhouses, *Salmonella* was isolated from the slaughter line before the onset of slaughter. Especially the conveyer belt and shackles in the dirty zone, the plucking machine and the scalding tank were contaminated. In these two slaughterhouses, the carcasses of the first slaughtered flock became contaminated with the same strains as isolated from the slaughter line before processing. This implies that slaughter of logistic slaughter, as applied in Belgium, is only successful when the cleaning and disinfection process completely eliminates the *Salmonella* contamination of the slaughter line. Only if this is achieved, will the slaughter of *Salmonella*-free flocks result in the absence of *Salmonella* on the carcasses.

Chapter VI describes a study in which the possible external contamination or colonization of broiler flocks was investigated during transport in containers still contaminated with *Campylobacter* after the cleaning and disinfection process. Indeed, it was shown that 25 of the 35 (71%) examined containers were contaminated with campylobacters at the moment of loading the birds. In total, 30 different genotypes were found in these containers with a maximum of four genotypes per individual container. After transport of flocks in such containers, no intestinal colonization of the flocks by campylobacters present in the transport containers was observed, though this transport led to limited external contamination with *Campylobacter.*

In conclusion, this doctoral thesis has demonstrated that the contribution of the gastrointestinal colonization to *Salmonella* carcass contamination during slaughter was low, whereas cross-contamination was high. Improperly cleaned and disinfected slaughter equipment was an important source for carcass contamination. For *Campylobacter*, the contribution of the gastrointestinal colonization to carcass contamination was high, whereas

cross-contamination was limited. The majority of the containers used to transport flocks were contaminated with *Campylobacter* and led to limited external contamination of the flocks.

SAMENVATTING

Salmonella en *Campylobacter* zijn twee belangrijke bacteriële oorzaken van gastro-enteritis in de westerse wereld. In 2005 werden in België 48 gevallen van *Salmonella* infectie en 66 gevallen van *Campylobacter* infectie per 100 000 inwoners geregistreerd. Voor beide bacteriën wordt de bewerking of consumptie van gecontamineerd kippenvlees beschouwd als een belangrijke oorzaak van infectie. In de meeste studies wordt nagegaan hoe het aantal kippentomen gekoloniseerd met *Salmonella* en *Campylobacter* kan gereduceerd worden op boerderijniveau om op die manier het risico op humane besmetting via kippenvlees te verlagen. Bepaalde studies hebben aangetoond dat kippenkarkassen na het slachtproces gecontamineerd waren met *Salmonella* en *Campylobacter*, terwijl de tomen op de boerderij vrij waren van deze pathogenen. Het doel van dit doctoraalwerk was de rol van het transport en het slachtproces op de contaminatie van pluimveekarkassen met *Salmonella* en *Campylobacter* te bestuderen met behulp van moleculaire technieken.

In de literatuurstudie wordt een overzicht gegeven van de taxonomie en karakteristieken van *Salmonella* en *Campylobacter.* Aansluitend worden de klinische aspecten, de epidemiologie en de besmettingsbronnen van *Salmonella* en *Campylobacter* infecties besproken. Vervolgens worden besmettingsroutes voor pluimvee, toomprevalentie, het Belgische slachtproces en de contaminatie van karkassen tijdens het slachtproces besproken. Tot slot worden de meest gebruikte moleculaire technieken voor de karakterisatie van *Salmonella* en *Campylobacter* isolaten beschreven.

In Hoofdstuk I wordt een vergelijking gemaakt van vijf rep-PCR gebaseerde technieken om *Salmonella* isolaten tot op serotype niveau te discrimineren. Op basis van het aantal bandjes, de uniforme verdeling van bandjes over het volledige bandenpatroon en de helderheid van de bandjes, werden de ERIC primer set en de $(GTG)_5$ primer gekozen om te evalueren in verdere experimenten. De reproduceerbaarheid werd getest op verschillende lysaten van vijf *Salmonella* serotypes tussen verschillende PCR runs in hetzelfde PCR toestel. De reproduceerbaarheid van de bekomen profielen was laag tussen verschillende PCR runs, maar hoog binnen éénzelfde PCR run. Verder werden profielen van 80 verschillende serotypes en vijf niet-serotypeerbare isolaten vergeleken. Alle stammen waren typeerbaar en hadden unieke profielen, met uitzondering van enkele stammen met een onvolledige antigenische code. Ten slotte werd de genetische diversiteit van genetisch verschillende stammen behorend tot eenzelfde serotype onderzocht. Een serotype correspondeerde niet altijd met slechts één ERIC of $(GTG)_5$ profiel, maar de heterogeniteit van de profielen binnen één serotype was

beperkt. Aangezien de reproduceerbaarheid, het discriminerend vermogen en de genetische diversiteit vergelijkbaar waren voor de ERIC primer set en de $(GTG)_5$ primer, zijn beide even bruikbaar in het discrimineren van *Salmonella* serotypes. In de studies beschreven in hoofdstuk II en V, werd de ERIC primer set gebruikt om het aantal te serotyperen *Salmonella* isolaten te reduceren.

In Hoofdstuk II wordt de bijdrage van gastro-intestinale kolonisatie en kruisbesmetting in de contaminatie van karkassen met *Salmonella* tijdens het slachtproces beschreven. Slechts 13% van de tomen braadkippen was gekoloniseerd met *Salmonella* op moment van slachten. Na het slachten waren karkassen van 55% van deze tomen gecontamineerd met *Salmonella*. Bij de leghennen en ouderdieren was 69% van de onderzochte tomen gekoloniseerd. Na slachten waren karkassen van alle tomen gecontamineerd met *Salmonella*. Moleculaire typering van de isolaten toonde aan dat de meeste stammen niet afkomstig waren van de gastro-intestinale inhoud van de tomen geslacht op de dag van staalname. Kruisbesmetting was in verschillende slachthuizen een groot probleem, onafhankelijk of de stammen afkomstig waren van de gastro-intestinale inhoud of van onduidelijke oorsprong. Bepaalde stammen werden geïsoleerd van alle tomen geslacht op dezelfde dag. Kruisbesmetting werd niet alleen geobserveerd naar volgend geslachte tomen maar ook naar tomen die voorafgaand waren geslacht.

In Hoofdstuk III wordt de bijdrage van gastro-intestinale kolonisatie en kruisbesmetting in de contaminatie van karkassen met *Campylobacter* tijdens het slachtproces beschreven. Op moment van slachten was 72% van de bemonsterde tomen gekoloniseerd met *Campylobacter.* Na het slachten waren karkassen van 79% van deze tomen gecontamineerd met *Campylobacter*. De isolaten werden geïdentificeerd als *Campylobacter jejuni* (89%), *Campylobacter coli* (8.7%) en *Campylobacter lari* (2.3%). Deze species werden verder onderverdeeld in 27 genotypes. Meestal waren de genotypes van de stammen geïsoleerd van karkassen van een toom dezelfde als deze geïsoleerd uit het gastro-intestinaal stelsel van dezelfde toom. Bij zes tomen (15%) werden stammen geïsoleerd van de karkassen die teruggevonden werden in de gastro-intestinale inhoud van kippen behorend tot één of twee voorafgaand geslachte tomen. Al was kruisbesmetting beperkt, vier van deze zes tomen waren niet gekoloniseerd met *Campylobacter* op moment van slachten en zouden *Campylobacter* vrij kunnen gebleven zijn na logistiek slachten.

Hoofdstuk IV omvat twee luiken. In de eerste plaats werd een mogelijke associatie tussen de gelijktijdige kolonisatie van tomen met *Salmonella* en *Campylobacter* onderzocht. Ten tweede werd bepaald welke deel van het gastro-intestaal stelsel aangewezen is voor bemonstering om de *Salmonella* en *Campylobacter* status van een toom te bepalen op slachthuisniveau. Stalen werden genomen van drie verschillende delen van het gastro-intestinaal stelsel, nl. de krop, de dunne darm en de blinde darmen. Er werd geen associatie tussen de gelijktijdige kolonisatie van tomen braadkippen met *Salmonella* en *Campylobacter* aangetoond. Aangezien alle tomen legkippen en ouderdieren gekoloniseerd waren met *Campylobacter* kon deze associatie niet onderzocht worden. Tot slot werd er aangetoond dat *Salmonella* en *Campylobacter* het vaakst geïsoleerd werden uit de dunne darm, gevolgd door de blinde darmen en tenslotte de krop. Staalname van de dunne darm was in deze studie voldoende om de *Campylobacter* status te bepalen. Daarentegen was het noodzakelijk de drie verschillende delen van het gastro-intestinaal stelsel te bemonsteren om alle tomen gekoloniseerd met *Salmonella* te identificeren.

In de studie beschreven in hoofdstuk V werd de impact van *Salmonella,* aanwezig op de slachtlijn, op de karkascontaminatie bepaald. In twee van de drie bemonsterde slachthuizen werd *Salmonella* aangetoond op de slachtlijn voor de opstart van de slachtactiviteiten. De schakelketting in de vuile zone, de plukmachine en de broeibak waren het meest gecontamineerd. In deze twee slachthuizen waren de karkassen van de eerst geslachte toom gecontamineerd met dezelfde stammen als voordien geïsoleerd van de slachtlijn. Deze resultaten tonen aan dat logistiek slachten, zoals toegepast in België, enkel zinvol is wanneer het reinigings- en desinfectieproces alle *Salmonella* bacteriën elimineert. Enkel wanneer dit bereikt wordt, zal het slachten van tomen met een *Salmonella*-vrije status resulteren in *Salmonella*-vrije karkassen.

Hoofdstuk VI tenslotte beschrijft een studie waarin onderzocht werd of tomen uitwendig gecontamineerd of gekoloniseerd kunnen worden tengevolge van transport van kippen in containers die na het reinigings- en desinfectieproces nog gecontamineerd waren met campylobacters. Er werd aangetoond dat 25 van de 35 onderzochte containers (71%) gecontamineerd waren met campylobacters op het moment dat de kippen geladen werden. In het totaal werden 30 verschillende genotypes uit deze containers geïsoleerd met een maximum van 4 genotypes per container. Na transport van tomen in dergelijk gecontamineerde containers, waren de kippen niet gekoloniseerd met *Campylobacter*

stammen afkomstig uit de transport containers, al waren de kippen occassioneel wel uitwendig gecontamineerd.

In dit doctoraalwerk is er aangetoond dat de gastro-intestinale bijdrage in de contaminatie van karkassen met *Salmonella* tijdens het slachtproces beperkt was. De karkassen werden voornamelijk gecontamineerd tengevolge van kruisbesmetting afkomstig van de slachtlijn. De contaminatie van karkassen met *Campylobacter* werd vooral veroorzaakt door het lekken van de gastro-intestinale inhoud tijdens het slachten. De meeste transport containers waren op moment van laden gecontamineerd met *Campylobacter*. Dit gaf aanleiding tot beperkte uitwendige besmetting van de tomen getransporteerd in deze containers.