Epidemiology of *Campylobacter* in Poultry

Epidemiologie van *Campylobacter* bij Pluimvee

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Epidemiology of *Campylobacter* in Poultry

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I

De belangrijkste *Campylobacter* besmettingsroute voor pluimvee is een horizontale overdracht vanuit de omgeving. *Dit proefschrift*

II

Behandeling met quinolonen is niet geschikt om het probleem van *Campylobacter* besmetting bij pluimvee op te lossen. *Dit proefschrift*

III

De hoge besmettingsgraad van Nederlandse tarwe met *Fusarium* (*Snijders, 1990; Netherlands Journal of Plant Pathology 96: 187-198*) vereist nader onderzoek naar de aanwezigheid van -voor vleeskuikens- schadelijke toxinen.

IV

De in de microbiologie veelvuldig gebruikte omschrijving "micro-aero**fiele** materialen en methoden" (*bijv. Smibert, 1984; In: Bergey's manual of systematic bacteriology, Vol. 1, p. 111-118*) is taalkundig gezien onjuist.

V

Er zijn zoveel mogelijkheden voor een vleeskuiken om met *Campylobacter* besmet te raken, dat een eventuele "levensvatbare niet-kweekbare vorm" (*Stern et al., 1994; Letters in Applied Microbiology 18: 333-336*) alleen daarom al weinig kans maakt om als werkelijk belangrijke besmettingsbron te worden aangemerkt.

VI

De beoordeling van manuscripten op geschiktheid voor publikatie in een wetenschappelijk tijdschrift zou standaard "double-blind" moeten plaatsvinden, zodat niet alleen de referenten maar ook de auteurs hierbij anoniem blijven.

VII

Om het inzicht van de consument in de houdbaarheid van levensmiddelen te vergroten verdient het aanbeveling om naast de momenteel voorgeschreven THT datum ook de produktiedatum op de verpakking te vermelden.

VIII

Het electro-cardiogram van gezonde mensen bevat meer informatie dan in het algemeen door medici wordt onderkend (*bijv. Dekker et al., 1994; Circulation 90: 779-785*).

IX

Aangezien een toxoplasmose-besmetting juist in de eerste maanden van de zwangerschap de meeste schade kan berokkenen, verdient het aanbeveling om de informatie hierover op de bijsluiter van zwangerschapstesten te vermelden.

X

Er rijden nog steeds te veel fietsers in auto's rond. *naar: Loesje*

Stellingen behorende bij het proefschrift van Wilma Jacobs-Reitsma:
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ABSTRACT

Campylobacter, causing human infections with severe symptoms of diarrhoea, is mainly transmitted by food, especially poultry meat products.
Several studies on *Campylobacter* colonization in breeders, laying hens, and broilers were carried out. *Campylobacter* isolates were serotyped, using a modification of the Penner system, in order to identify epidemiological factors contributing to the *Campylobacter* colonization of poultry. No evidence was found for vertical transmission from breeder flocks via the hatchery to progeny, nor for a horizontal transmission from one broiler flock to the next via a persistent house-contamination. By far the major route for *Campylobacter* contamination of poultry is a horizontal transmission from the environment. Pigs and poultry flocks (broilers, as well as laying hens and breeders), and to a lesser extent, sheep and cattle were found to be potential sources of *Campylobacter* contamination. Horizontal intervention procedures at the farm level have to be studied further to evaluate the effectiveness of strict hygienic practices during the whole production period.
A screening for antibiotic resistance revealed 181 out of 617 *Campylobacter* isolates (29%), originating from a large number of broiler flocks, to be quinolone-resistant. Quinolone treatment of *Campylobacter* colonized broiler chicks was found to induce quinolone resistance in the *Campylobacter* bacteria under experimental conditions. Therefore, quinolone treatment should not be seen as an answer to the problem of eradication of *Campylobacter* contamination from poultry flocks.

foar Heit en Mem

vur Cörke

Voorwoord

Beste

Bart, Frans (F.F.P.), Henk, Kees, Marga, Nico, Ria, Roel;
Antonio, Carla, Debbie, Hannah, Ivo;
Frans R., Petra (M.F.J.), Wilma;
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Annelies en de overige medewerkers van de proevendienst;
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Erica, Sigi;
Cor;
en al die anderen die op een of andere manier aan dit proefschrift hebben bijgedragen;

Tige Tank !

ek mei groetnis fan .....

Contents

CHAPTER 1

Introduction

Campylobacter infections in humans

Campylobacter bacteria are recognized as one of the most important causes of human diarrhoeal disease. Generally, a *Campylobacter* infection in humans (campylobacteriosis) manifests itself as a self-limiting enteritis. Symptoms like fever, diarrhoea, nausea and characteristic abdominal cramps generally last for about two to seven days. The faecal excretion of the organism among untreated patients lasts for two or three weeks. A prolonged carrier state is rare. Medication of uncomplicated cases generally is not necessary. If antibiotics are prescribed, erythromycin usually is the drug of choice. (Walker et al., 1986; Skirrow and Blaser, 1992). Recently, fluoroquinolones have been used, sometimes associated with prophylactic use in travellers' diarrhoea (Hirschl et al., 1990; Rademaker et al., 1989; Mattila et al., 1993).

The infectious dose of *Campylobacter jejuni* for humans appears to be low. Several studies with volunteers showed that the relatively low ingestion of 500-800 cells was sufficient to cause illness in humans (Robinson, 1981; Black et al., 1988).

A consistent seasonal distribution of human *Campylobacter* infections has been reported for several years in the temperate climates of both Europe and USA. A sharp rise is seen in May, followed by a peak in July and August. A second, but smaller, peak is observed in autumn (Skirrow, 1987; Hoogenboom-Verdegaal et al., 1990; Tauxe, 1992).

The incidence of *Campylobacter* infections in humans is not easily estimated. Dutch regional hospital laboratories isolated *Campylobacter* bacteria from 12-15% of patients with acute enteritis during the period 1987-1992 (Notermans and Van de Giessen, 1993). Laboratory reports, however, represent only a small part of all infections. Results from a Dutch population study at the community level indicated that about 10% of the patients with campylobacteriosis consulted their physician (Hoogenboom-Verdegaal et al., 1992). Based on this information, the true incidence of *Campylobacter* infections was estimated to be 18-23 cases per 1000 per year. Calculated for the total Dutch population, 300,000 cases of *Campylobacter* infection may occur annually in The Netherlands. In the same study, *Salmonella* was estimated to account for some 130,000 cases per year (Notermans and Van de Giessen, 1993). Apart from the human suffering, the economic impact due to lost productivity and health care of this number of *Campylobacter* infections is considerable.

Sources of human campylobacteriosis

Campylobacteriosis in man is mainly a food-borne infection, in which foods of animal origin, and in particular poultry products, play an important role. Large community outbreaks are rare, but several occasions were reported to be caused by consumption of untreated surface water or raw milk. The majority of *Campylobacter* infections are sporadic (single) cases or small family outbreaks (Blaser et al., 1983; Griffith and Park, 1990; Tauxe, 1992). In several case-control studies of sporadic campylobacteriosis, over 50% of the cases were attributed to the consumption of (undercooked) poultry meat (Oosterom et al., 1984; Harris et al., 1986). Also handling of raw poultry was found to be a risk factor (Hopkins and Scott, 1983; Deming et al., 1987).
Infection can also be acquired by direct contact with infected animals, mainly in particular situations like for the workers in poultry processing plants (Jones and Robinson, 1981; Christenson et al., 1983). Juvenile dogs and cats or pets with diarrhoea are frequently found to be carriers of the organism and contact with these animals may lead to infection (Deming et al., 1987; Tauxe, 1992).
In The Netherlands, poultry products are considered to be the main source of food-borne *Campylobacter* infections in humans (Health Council of The Netherlands, 1988).

Campylobacter in animal reservoirs

Wild birds and domestic animals are regarded as being the main reservoir of *Campylobacter* in the environment. These warm-blooded animals often carry large numbers of *Campylobacter* bacteria in their intestinal tracts, mostly without showing any symptoms of disease. During slaughter of meat producing animals like poultry, pigs, cattle or sheep, intestinal contents may spread on the carcasses and thus cause contamination of end-products. At the retail level, 40-100% of the poultry meat products is found to be contaminated with *Campylobacter* (Shane, 1992; Van der Zee et al., 1994), whereas *Campylobacter* contamination of pork or beef is found to be much lower (Stern et al., 1985; Lammerding et al., 1988). This difference in contamination rate is thought to be associated with the differences in

the way of chilling the various meat type carcasses and the differences in skin structure (Oosterom et al., 1983b). Both the sensitivity of *Campylobacter* for drying (Oosterom et al., 1983b; Bracewell et al., 1985), as well as the oxygen sensitivity of the organism (Bolton et al., 1982) were suggested to play a role.
Like other enteric bacteria, *Campylobacter* is sensitive to heat and will be eliminated when the contaminated product is heated thoroughly. Problems arise when undercooked meat is consumed (as is likely to happen at a barbecue) or when raw meat is handled improperly and cross-contamination to other foods can occur (Skirrow and Blaser, 1992).

Campylobacter epidemiology in poultry

Since poultry products account for the majority of *Campylobacter* infections in humans, reduction of the *Campylobacter* contamination of these products will have the greatest impact on this human health problem. Because of the low infectious dose of *Campylobacter*, it will probably be more effective to reduce the total number of contaminated carcasses, rather than to reduce the number of campylobacters on each carcass. Therefore, prevention of *Campylobacter* colonization in broiler flocks at the farm level is the most elegant and efficient way to reduce the contamination level of poultry products.
Detailed information on the transmission routes by which *Campylobacter* colonizes poultry is needed for the formulation of adequate intervention strategies at the broiler farm level. Studies to identify sources and to trace transmission routes are necessary, but require detailed identification of *Campylobacter* spp. isolates in order to give adequate information. Several typing systems, including biotyping, serotyping, phagetyping and typing systems based on DNA analysis, have been developed to support these epidemiological investigations (Patton and Wachmuth, 1992; Jorgensen, 1993). The serotyping system described by Penner and Hennessy (1980) is based on soluble heat-stable antigens and a passive haemagglutination technique. It is one of the best known and widely used typing systems for *Campylobacter* spp. (Patton and Wachmuth, 1992).
Various studies on *Campylobacter* epidemiology in poultry have been described (Evans, 1992; Stern, 1992; Van de Giessen et al., 1992; Kapperud et al., 1993).

Up to now, a number of possible factors involved in *Campylobacter* colonization of broilers have been suggested. These factors are represented in the flow diagram in Figure 1.1.

At the start, the broiler house is assumed to be *Campylobacter*-free, although the possibility of transmission of *Campylobacter* from one broiler production cycle to the next one has to be considered.

One-day-old chicks then enter the *Campylobacter*-free broiler house and might be the carriers of a vertical *Campylobacter* transmission from their parent flock or the hatchery.

Feed, water and air are some of the continuous horizontal flows into the broiler house. Chicken transport crates (and the associated people) are a transmission factor

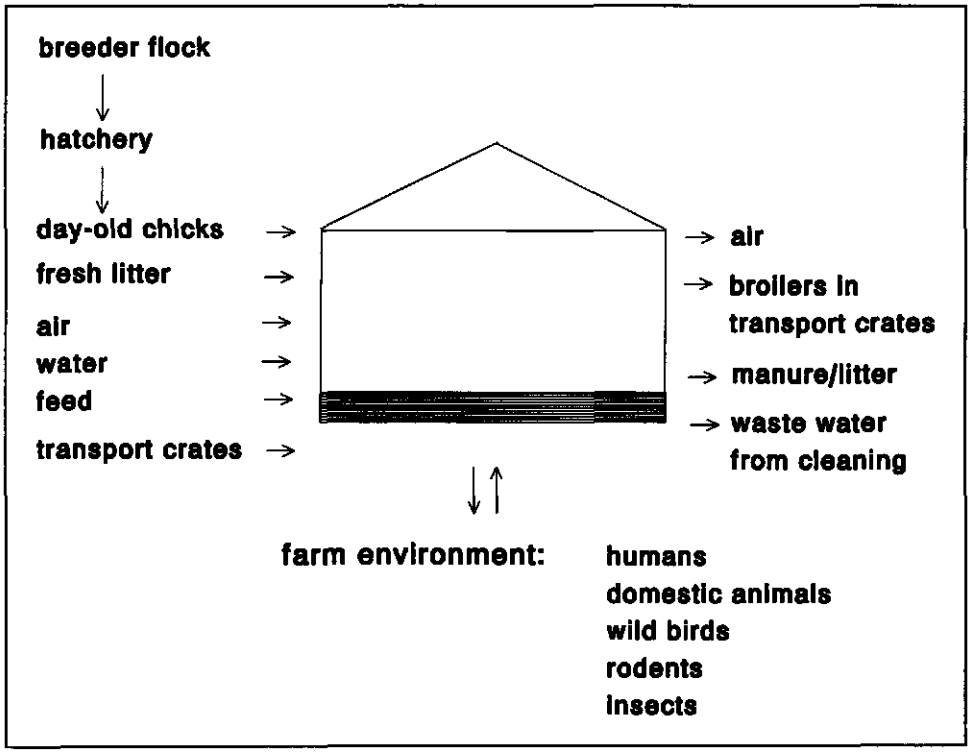

Figure 1.1. *Flow diagram of possible* Campylobacter *transmission routes in a broiler house.*

at the end of the production cycle. Under Dutch husbandry conditions, this might happen at the broiler age of about five weeks, when some 25% of the broilers are taken out, and subsequently at about six weeks of age when the remainder of the flock is taken to slaughter.

Several environmental sources of *Campylobacter*, such as domestic animals, insects, and rodents, may be present on the farm. The farmer, coming in the broiler house daily, may serve as "the vehicle of transmission" from these environmental sources. Moreover, some sources (insects, rodents) do not necessarily need a (human) carrier as they are able to enter the house themselves and cause *Campylobacter* contamination directly.

The broilers leave the house at five or six weeks of age, and afterwards litter is removed, the house is totally emptied, cleaned and disinfected before another production cycle starts.

The broiler chickens themselves, but also the used litter and the waste water from cleaning, might be important in supporting and maintaining the external environmental contamination cycles of *Campylobacter*.

Another aspect of *Campylobacter* epidemiology in poultry was disclosed by Endtz et al. (1991). They reported on the quinolone resistance of *Campylobacter* strains isolated in The Netherlands between 1982 and 1989 from poultry products and human stools. The prevalence of resistant strains isolated from poultry products increased over these years from 0% to 14% and the prevalence in human isolates increased from 0% to 11%. The rise in quinolones resistant *Campylobacter* isolates in both humans and poultry products was suggested to be related to the extensive use of quinolones in veterinary practice.

Emergence of quinolone resistant *Campylobacter* isolates was also reported for other European countries (Rautelin et al., 1991; Reina et al., 1992). This rapid increase in quinolone resistant bacteria may have implications for the treatment and prophylaxis of human diarrhoeal disease.

Aim and organization of this thesis

The aim of the work presented in this thesis is to obtain a better understanding of the *Campylobacter* epidemiology in poultry, in order to be able to formulate

recommendations to reduce *Campylobacter* colonization in live broiler flocks. In this thesis several studies on various epidemiological aspects as illustrated in Figure 1.1 are described.

The Penner serotyping system was used as the epidemiological marker in most of the studies reported in this thesis. Several modifications on the original method described by Penner were introduced and are presented in Chapter 2.

A screening study on *Campylobacter* colonization in breeder flocks is described in Chapter 3.

Chapter 4 deals with *Campylobacter* epidemiology in laying hens and their possible role in *Campylobacter* transmission routes.

Chapter 5 describes a longitudinal survey on two broiler farms, where serotyping was used to identify possible *Campylobacter* transmission routes.

A one-year study on seasonal prevalence of *Campylobacter* and *Salmonella* in caecal samples of broilers at slaughter is described in Chapter 6. Data on the sampled flocks were studied to identify possible factors for *Campylobacter* colonization of broiler flocks.

A screening for antibiotic resistance, especially to quinolones, was included in the one-year study on the prevalence of *Campylobacter* and *Salmonella* at slaughter. The antibiotic resistance results are presented in Chapter 7.

Results of a study on the induction of quinolone resistance in *Campylobacter* bacteria in broilers by quinolone treatment are described in Chapter 8.

Finally, a general discussion of the work presented in this thesis is given in Chapter 9.

CHAPTER 2

Penner Serotyping of *Campylobacter* Isolates from Poultry, Using Pools of Absorbed Antisera

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ABSTRACT The Penner serotyping system, based on detection of heat-stable antigens with a passive haemagglutination technique, was used in several studies on *Campylobacter* epidemiology in poultry. The modifications of using absorbed and pooled antisera were included. Over 80% of the *Campylobacter* isolates in our studies were typable with this modified Penner serotyping system. Typability of strains was clearly affected by storage of the strains before actual typing. As extracted antigens appeared to be stable for at least six months at 4 °C, it is advisable to store extracted antigens from freshly isolated *Campylobacter* strains instead of reculturing frozen-stored strains, when actual typing can not be performed directly after primary isolation. Untypability of isolates may partly be explained by the detection of *Campylobacter* serotypes not yet represented in the serotyping system. Experiments on repeated serotyping of several *Campylobacter* strains did not suggest any serotype instability within the strains.

Introduction

Campylobacter spp. are recognized as one of the most important causes of human diarrhoeal illness. Several case-control studies have implicated that handling and consumption of (undercooked) poultry meat are the major sources of human infection (Griffith and Park, 1990; Skirrow and Blaser, 1992). A reduction in the number of *Campylobacter* infections transmitted to humans by poultry may be best achieved by prevention of *Campylobacter* colonization at the broiler house level (Stern, 1992; Evans, 1992). Epidemiological studies to identify sources and to trace transmission routes in poultry are carried out, but require subtyping of *Campylobacter* spp. isolates. Several serotyping systems have been developed to support these epidemiological investigations (Penner and Hennessy, 1980; Lauwers et al., 1981; Lior et al., 1982). The system described by Penner is based on soluble heat-stable antigens and a passive haemagglutination technique (PHA) and is one of the best known and widely used typing systems for *Campylobacter* spp. (Patton and Wachmuth, 1992).
A modified Penner serotyping system was used in several studies on *Campylobacter* epidemiology in poultry. Antisera were absorbed to increase the specificity and reduce the number of cross-reactions. Use of absorbed antisera also allowed the use of pools of antisera, which may save labour, time and materials in the serotyping of routine samples.

Materials and methods

Bacteria

Penner reference strains 1 to 65 (Table 2.1) were obtained from the Culture Collection of the University of Göteborg (CCUG), Sweden, and included 47 *C. jejuni* and 18 *C. coli* strains. Reference strains were stored at -80 °C in cryovials (Microbank, Pro-Lab Diagnostics).
Campylobacter isolates originated from the following studies on *Campylobacter* epidemiology in poultry: a study on broiler breeders (Jacobs-Reitsma, 1994f); a study on two broiler farms including other domestic animals (Jacobs-Reitsma et al.,

1994e); a study on turkeys (Jacobs-Reitsma, 1992b); a study on a sewage plant and a connected poultry slaughterhouse (Koenraad et al., 1994); and a study on laying hens (Jacobs-Reitsma, 1994g). Strains were tested either directly after primary isolation and purification or after storage at -80 °C.

Preparation of antisera

Penner reference strains were micro-aerobically grown on Columbia agar plates (Oxoid CM 331) with 5% horse blood and *Campylobacter* growth supplement (Oxoid SR 84). The growth from one plate was transferred to 10 ml of phosphate-buffered saline, pH 7.4 (PBS). In the case of formalin-treated cells (used on day 1 and day 6 as described below), the growth was harvested in PBS containing 0.5% formalin and kept at room temperature for 24 and two hours, respectively. All cell suspensions were washed twice with PBS and resuspended in PBS at a concentration of $0.5 \cdot 10^9$ colony forming units per ml, before inoculation of the rabbits.

New Zealand white rabbits weighing about 2500 g were immunized intravenously with 0.25 ml formalin-treated antigen on day 1, with 0.50 ml formalin-treated antigen on day 6, with 1.0 ml live antigen on day 11 and with 2.0 ml live antigen on day 16. The rabbits were bled at day 21 and antisera were separated and stored at -20 °C.

Absorption of antisera

Antisera were tested for specificity by titration against all 65 reference strains, including the autologous strain. Cross-reacting antisera were made specific by absorption with the cross-reacting antigens. After trials to establish the quantity of bacterial cells required, antisera were absorbed by overnight incubation of antiserum with a freshly prepared cell suspension of the reference strain at room temperature with gentle shaking. Cell material was removed by centrifugation and absorbed antiserum was tested again for specificity; if necessary, absorptions were repeated. Absorptions are listed in Table 2.2.

Table 2.1. Specifications of Penner reference strains and the antigenic structure as determined in this study.

CCUG number	Species	Penner serotype	Antigen structure
10935	C. jejuni	1	1,44
10936	C. jejuni	2	2
10937	C. jejuni	3	3
10938	C. jejuni	4	4,50
15359	C. jejuni	5	5
12778	C. jejuni	6	6,7
10940	C. jejuni	7	7
16436	C. jejuni	8	8
10942	C. jejuni	9	9
10943	C. jejuni	10	10
10944	C. jejuni	11	11
17625	C. jejuni	12	12
10945	C. jejuni	13	13,50
15360	C. coli	14	14
10946	C. jejuni	15	15
10947	C. jejuni	16	16
10948	C. jejuni	17	8,17
10949	C. jejuni	18	18
10950	C. jejuni	19	19
10951	C. coli	20	20
10952	C. jejuni	21	21
10953	C. jejuni	22	22
10954	C. jejuni	23	23,36
10955	C. coli	24	24,54
10956	C. coli	25	6,7
10957	C. coli	26	26
10958	C. jejuni	27	27
10959	C. coli	28	28
15361	C. jejuni	29	29
10960	C. coli	30	30
10961	C. jejuni	31	31
10962	C. jejuni	32	32
10963	C. jejuni	33	33

(Continued)

Table 2.1. (Continued).

CCUG number	Species	Penner serotype	Antigen structure
10964	C. coli	34	34
10965	C. jejuni	35	35
10966	C. jejuni	36	36
10967	C. jejuni	37	37
10968	C. jejuni	38	38
10969	C. coli	39	39
10970	C. jejuni	40	38,42
10971	C. jejuni	41	41
12782	C. jejuni	42	42
12783	C. jejuni	43	43,50
14567	C. jejuni	44	44
17753	C. jejuni	45	45
15362	C. coli	46	46
17715	C. coli	47	47
17754	C. coli	48	48
17755	C. coli	49	49
12790	C. jejuni	50	13,50
12791	C. coli	51	51
12792	C. jejuni	52	52
15013	C. jejuni	53	53
12794	C. coli	54	54
12795	C. jejuni	55	55
14537	C. coli	56	56
14538	C. jejuni	57	57
14539	C. jejuni	58	58
17626	C. coli	59	59
14541	C. jejuni	60	60
24865	C. coli	61	61
24866	C. jejuni	62	62,64
24867	C. jejuni	63	63
24868	C. jejuni	64	64
24869	C. jejuni	65	64,65

Table 2.2. Antisera, absorptions, results and pools.

Antiserum	Absorptions	Result	Pool
O1,44	-	O1,44	DE
	O44	O1	*
O2	-	O2	FG
O3	O29	O3	HI
O4,50	O50	O4,50	AI
	O13,50; O59	O4	*
O5	-	O5	EG
O6,7	-	O6,7	AC
	O7	O6	*
O7	-	O7	*
O8	-	O8	*
O9	-	O9	EF
O10	-	O10	GH
O11	-	O11	DK
O12	-	O12	LK
O13,50	-	O13,50	AI
	O4,50	O13	*
O14	-	O14	BH
O15	O29	O15	CD
O16	O22	O16	FH
O8,17	-	O8,17	BD
	O8	O17	*
O18	O17; O29	O18	FG
O19	-	O19	EI
O20	-	O20	GK
O21	-	O21	FL
O22	-	O22	AE
O23,36	-	O23,36	BC
	O36	O23	*
O24,54	-	O24,54	DG
	O54	O24	*
O26	O34; O62	O26	HK
O27	O23	O27	IL
O28	-	O28	CM
O29	O21; O38	O29	DH

(Continued)

Table 2.2. (Continued).

Antiserum	Absorptions	Result	Pool
O30	O34; O45	O30	AM
O31	O5	O31	AG
O32	-	O32	BF
O33	O32	O33	IM
O34	O26	O34	AD
O35	-	O35	BG
O36	O29	O36	*
O37	O56	O37	CE
O38	-	O38	BM
O39	O23; O29	O39	FK
O41	-	O41	HL
O42	O29	O42	BM
O43,50	-	O43,50	AI
	O4,50	O43	*
O44	-	O44	DE
O45	-	O45	AM
O46	-	O46	BK
O47	O30; O45	O47	CI
O48	-	O48	DM
O49	-	O49	EH
O51	O26	O51	FI
O52	O13,50	O52	GL
053	-	O53	EK
O54	-	O54	*
O55	O42	O55	AK
O56	O37	O56	BL
O57	-	O57	FM
O58	-	O58	CG
O59	O3; O4,50; O8,17	O59	DF
O60	-	O60	DI
O61	-	O61	HM
O62,64	O16	O62,64	CK
O63	-	O63	AB
O64	O59	O64	*
O64,65	O13,50	O64,65	CK

* specific antisera not in pools, only used in titration

Antiserum pools

The antisera were divided over 12 pools (A - M, Table 2.3) in such a way that each strain could be recognized by a combination of a two-letter code (Table 2.2). No differentiation was made between *C. jejuni* and *C. coli* in the composition of the pools. The antiserum dilution used depended upon the titres of the autologous strain in the antiserum. Antisera with titres of 1:1280 or 1:2500 were diluted 250 times, those with titres of ≥ 1:5000 were diluted 1000 times.

Preparation of antigens and sensitization of erythrocytes

Isolates were grown microaerobically (CampyPak Plus, BBL 71045) on blood agar plates (No.2, Oxoid CM271) with 5% sheep blood for 48 hours at 37 °C. Cells were harvested in 5 ml PBS and heated for one hour at 100 °C. After centrifugation, the supernatant containing the heat-stable antigens was kept at 4 °C.
Sheep erythrocytes (washed 1% suspension in PBS) were sensitized with the PBS-extracted antigens by incubating equal volumes (0.5 ml) for one hour at 37 °C in a

Table 2.3. Composition of pools.

Pool	O Antisera										
A	4,50	13,50	43,50	6,7	22	31	30	34	45	55	63
B	8,17	23,36	14	32	35	38	42	46	56	63	
C	6,7	23,36	62,64	64,65	15	28	37	47	58	65	
D	1,44	8,17	24,54	11	29	34	44	48	59	60	
E	1,44	5	9	19	22	37	49	53			
F	2	9	16	18	21	32	39	51	57	59	
G	24,54	2	5	10	18	20	31	35	52	58	
H	3	10	14	16	26	29	41	49	61		
I	4,50	13,50	43,50	3	19	27	33	47	51	60	
K	62,64	64,65	11	20	26	39	46	53	55		
L	12	21	27	41	52	56					
M	12	28	30	33	38	42	45	48	57	61	

gently shaking waterbath. The sensitized erythrocytes were centrifuged, washed twice in PBS, and resuspended in PBS to a final cell suspension of 0.5%. This suspension was stable for two days when kept at 4 °C.

Passive haemagglutination with pooled antisera

Microtitration plates with U shaped wells (Greiner 650101) were filled with 100 µl of pool solutions A-M, respectively. A volume of 100 µl of the sensitized erythrocyte suspension was added to each of the 12 wells containing the different pools. The plates were incubated at room temperature (20 °C) for one hour and subsequently for at least three hours at 4 °C. Plates were read by examining wells for agglutination of erythrocytes (positive reaction in two out of 12 wells each sample). The serotype was identified by the two-letter code thus produced (Table 2.2). The serotype was confirmed by titration of the single antiserum or in the case of a complex serotype, with the antisera mentioned in Table 2.4. When three pools were positive, each possible letter code was titrated. Strains positive in four or more pools were considered auto-agglutinable.

Passive haemagglutination titration with single antisera

Twofold dilutions of the single antiserum (used in a 1:10 dilution in PBS) were made in microtitration plate wells filled with 100 µl PBS. One well was used as a negative control, without antiserum. A volume of 100 µl of the sensitized erythrocyte suspension was added to the 12 wells and the plates were incubated and read as described above. The initial dilution of the antisera was 1:40. The titre was taken as the highest dilution in which a positive reaction occurred. A reaction was considered positive until two steps below the autologous titre of the antigen.

Stability of strain serotypes

A selection of *Campylobacter* strains was used to test the stability of the serotype and that of the extracted antigen. Fifteen strains of various serotypes were subcultured 10 times on blood agar plates and were serotyped (pools and single antiserum titration) every second subculturing. The primary extracted antigens were re-tested after storage at 4 °C for six months.

Table 2.4. Use of antisera for interpretation of serotypes.

Positive reaction	Antisera to be titrated	Serotypes found
DE = 1,44	1 and 44	O1; O44; O1,44
AI = 4,50	4,50; 13,50; 4; 13; 43	O4; O13; O43
= 13,50		O4,50; O13,50
= 43,50		O43,50
AC = 6,7	6 and 7	O6; O7; O6,7
BD = 8,17	8 and 17	O8; O8,17
BC = 23,36	23 and 36	O23; O36; O23,36
DG = 24,54	24 and 54	O24; O54; O24,54
AM = 30,45*	30 and 45	O30; O45
CK = 62,64	62,64; 64,65; 64	O62; O64; O65
= 64,65		O62,64; O64,65

* Titrations not due to relationship, but due to the composition of the pool.

Results

Absorption of antisera

Each crude antiserum was titrated against all reference strains. Autologous titres ranged from 1:1280 to 1:≥40,000. A number of cross-reactions were observed and absorptions were performed (Table 2.2).

Based on these data an antigenic structure was found as given in Table 2.1. This information was used to establish the composition of the pools.

Reference strains O13 and O50 were identical. O4, O13, O43, and O50 cross-reacted and were found to share an antigenic determinant. Reference strain O25 behaved similar as O6,7; strain O40 was composed of the antigens of strain O38 and O42. Reference strains O62 and O65 have the antigenic determinant O64. Several strains can be identified as single or complex-serotypes:

O1; O44 and O1,44
O4; O13; O43: O4,50; O13,50 and O43,50
O6; O7 and O6,7
O23; O36 and O23,36
O24; O54 and O24,54

Passive haemagglutination technique with pooled antisera

Generally, the PHA titration with the single antiserum confirmed the serotype as indicated from the PHA with the pooled antisera, and titres were sufficiently high (> 1:320 or > 1:1280).

Only one cluster of isolates showed an autoagglutination pattern, with a positive reaction in more than eight of the 12 pools. These isolates all originated from the same isolation source and were serotyped on the same day. After subculturing, these strains were shown to be serotype O30, without signs of autoagglutination.

A number of strains remained untypable in the PHA with pooled antisera. Occasionally, these strains were subcultured several times and re-tested, which often resulted in a typable isolate.

A single positive reaction in pool E only was observed from time to time. Fourteen isolates from various sampling sites were tested on all possible two-letter codes combinations with E (serotypes O22; O37; O1,44; O9; O5; O49; O19; O53). These strains usually were found in their two-letter code in pools. Seven of these isolates appeared to be of serotype O1,44 (reciprocal titres ranged from 80 - 2500), three isolates were O53 (reciprocal titres 1280 and 5000), two isolates were O37 (titres 1280) and two isolates remained untypable.

Simultaneous occurrence of two serotypes in one isolate was found several times, both serotypes giving a normal titre of > 1:1280. The combination of serotypes did not seem to be correlated to particular serotypes. Mostly, the two serotypes were also detected separately in samples from the particular sampling site.

Stability of serotypes

No changes in the original serotype were noticed in the serotype stability testing of 15 strains, though differences in titres were observed frequently. Strains COVP

2067 (O1,44) and COVP 990 (O28) appeared to be untypable in the pools in the last of the five tests, which had been stored at 4 °C for two days prior to extraction of the antigen. Titres were 80/1280 and 320, respectively.
Extracted antigens remained stable for at least six months at 4 °C, though a small drop in titre was noticed for some extracts.

Serotyping results of the various epidemiological studies

An overview of results obtained with this serotyping system in the various studies on the epidemiology of *Campylobacter* in poultry is given in Table 2.5. It should be noted that some studies included repeated sampling of a limited number of sampling sites, which may lead to an over-estimation of the prevalence of certain serotypes.
Nevertheless, the most commonly isolated serotypes were O1,44; O2; O24,54; O30; O37; O46; O53; O56; O59. No isolates of serotypes O4; O8; O10; O15; O17; O18; O19; O26; O27; O32; O35; O40; O41; O50; O52; O54; O55; O58; O61; O62 or O64 were found.
Overall, about 80% of the strains could be serotyped with the system using pools of absorbed antisera. The percentage of untypable isolates differed between the various sampling sources: all 30 turkey isolates could be serotyped (Table 2.5, *i*), whereas a total of 26 from 55 broiler isolates obtained at slaughter (47%) remained untypable (Table 2.5, j).

Discussion

The Penner serotyping system with absorbed and pooled antisera as described was easy to work with in routine samplings. However, the availability of antisera is crucial; when production and efficiency testing of new batches of absorbed antisera has to be included, the system is much more laborious.
False positive reactions in unexpected combinations of pools did not occur. Titration with the single antiserum always confirmed the suspected serotype, and titres generally were sufficiently high (> 1:320 or > 1:1280).
The possibility of cross-reactivity in reference strains known to be closely related (Penner et al., 1983; Patton et al., 1985) was avoided by taking this fact into

Table 2.5. Overview of serotyping results of several epidemiological studies in poultry.

	Origin of samples														
Serotype	a	b	c	d	e	f	g	h	i	j	k	l	m	Total	%
O1,44	6	1	22	6	1				1		3	9	24	73	4.5
O2	51	55	1	21	2		2	5		1	5	8	19	170	10.4
O3		1									1	2	20	24	1.5
O5	31					4	3					1	1	40	2.4
O6,7				1		4						2		7	0.4
O9									2		1	1		4	0.2
O11				15	1						8	5		29	1.8
O12										2	1			3	0.2
O13	2	2						1					25	30	1.8
O14		1	8						8		2			19	1.2
O16			5	3							1	1	3	13	0.8
O20						1								1	0.1
O21	4										1	1		6	0.4
O22												1		1	0.1
O23,36							1		1					2	0.1
O24,54	18	6	5			3							24	56	3.4
O28	13	6	2									2		23	1.4
O29										12	1			13	0.8
O30	75		1	13	1	5						2	20	117	7.1
O31	1													1	0.1
O33				3										3	0.2
O34		30		2										32	2.0
O37	22	18		62	1					1	6	1	5	116	7.1
O38,42	1									1		1		3	0.2
O39						1								1	0.1
O43													1	1	0.1
O46	9			23		2			9	5	4	1		53	3.2
O47	2											1		3	0.2
O48	9					2	1				1			13	0.8
O49		23												23	1.4
O51			1											1	0.1
O53	24	2	2	5	2							3	25	63	3.8
O56	1	121				8								130	7.9
O57	9			1								2		12	0.7
O59	5	4	7	141	19	1		3				4		184	11.2
O60		32	2						9					43	2.6
O63	4									7	2			13	0.8
O65			1	1									4	6	0.4
Non typable	43	28	7	54	17	1		3		26	17	16	96	308	18.8
Total	330	330	64	351	44	32	7	12	30	55	54	64	267	1640	100.0

a	breeders	1990	(Jacobs-Reitsma, 1994f)
b	broilers (farm D)	1989-1991	(Jacobs-Reitsma et al., 1994e)
c	laying hens (farm D)	1989-1991	(Jacobs-Reitsma et al., 1994e)
d	broilers (farm E)	1989-1991	(Jacobs-Reitsma et al., 1994e)
e	insects (farm E)	1989-1991	(Jacobs-Reitsma et al., 1994e)
f	pigs (farm E)	1989-1991	(Jacobs-Reitsma et al., 1994e)
g	sheep (farm E)	1989-1991	(Jacobs-Reitsma et al., 1994e)
h	cattle (farm E)	1989-1991	(Jacobs-Reitsma et al., 1994e)
i	turkeys	1992	(Jacobs-Reitsma, 1992b)
j	broilers at slaughter	1992/1993	(Koenraad et al., 1994)
k	slaughterhouse effluent	1992/1993	(Koenraad et al., 1994)
l	waste water plant	1992/1993	(Koenraad et al., 1994)
m	laying hens (flocks A)	1991/1992	(Jacobs-Reitsma, 1994g)

account in the composition of the pools. Titration of these closely related antisera sometimes yielded variable results, but no further attempts were made to distinguish within these antigen complexes.

No explanation was found for the observation of a positive haemagglutination reaction in only pool E.

Over 80% of the tested strains (Table 2.5) were typable with the system. Other studies using the heat stable serotyping system report typabilities of 74-100% for human isolates and 51-95% for animal isolates, also depending on the number of antisera that were available (Patton and Wachsmuth, 1992).

Typability of strains was clearly affected by storage of the strains before actual typing. All 30 turkey isolates were typed directly after primary isolation and were easily typed, whereas over 35% of broiler isolates at slaughter or isolates from laying hens after storage at -80 °C were untypable (Table 2.5). Additional subculturings on blood agar plates and re-testing of a number of these stored strains did in most cases result in a typable isolate. However, this leads to an undesirable extra effort in work and time.

As also reported by Fricker (1986) and Lastovica et al. (1986), extracted antigens appeared to be stable for at least six months at 4 °C. Therefore, it is advisable to store extracted antigens from freshly isolated *Campylobacter* strains instead of reculturing frozen-stored strains, when actual typing can not be performed directly after primary isolation.

Another explanation for a number of untypable strains is the detection of *Campylobacter* serotypes not represented in the serotyping system up to now. Indications for this were observed in both the study on two broiler farms (Table 2.5, b) and that on broiler flocks at slaughter (Table 2.5, j). Along with typable isolates, clusters of *Campylobacter* isolates, originating from one particular source, invariably remained untypable. These strains might be suitable for extending the typing system, as was done by Lastovica et al. (1986).

Two different serotypes were detected in one isolate from time to time. However, both serotypes could also be isolated separately from the same sampling site. This suggests more a problem in obtaining a single, pure *Campylobacter* colony for testing, rather than the occurrence of transient antigens as reported by Patton et al. (1985).

Testing the serotype stability of a number of strains did not indicate any shift in serotype. So, the large number of different serotypes isolated from and within the

various studies in Table 2.5 indicate the complexity of *Campylobacter* epidemiology in poultry in The Netherlands. Nevertheless, the Penner serotyping system, combined with the described modifications, was suitable for routine use and did provide valuable information in these epidemiological studies.

CHAPTER 3

Campylobacter and *Salmonella* in Breeder Flocks

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ABSTRACT Nine Dutch breeder farms with a total of 43 separately housed flocks were screened for the presence of *Campylobacter* spp. and *Salmonella* spp. Penner serotyping of *Campylobacter* isolates was used to identify epidemiological factors contributing to the *Campylobacter* infection of these breeder flocks. *Campylobacter* was isolated from 29 of the flocks (67%) and *Salmonella* from 12 flocks (28%). Two of the nine farms were both *Campylobacter* and *Salmonella*-free at the time of sampling. Two other farms were *Salmonella*-free, but were *Campylobacter*-positive. A total of 330 *Campylobacter* isolates were serotyped and 19 different *Campylobacter* serotypes were isolated in this study. A similar pattern of serotypes was generally observed in all flocks on a farm. *Campylobacter* colonization of breeder flocks implicates a potential role of vertical transmission via the egg to progeny. However, serotyping results do not support a vertical transmission route via the egg. Therefore, breeder flocks have to be recognized as another reservoir of *Campylobacter* and it is more appropriate to consider them as a potential risk factor in horizontal transmission routes.

Introduction

On the basis of sentinel and population studies, infections with *Campylobacter* have been estimated to occur in about 300,000 persons in The Netherlands each year (Notermans and Van de Giessen, 1993). These human cases of campylobacteriosis are most likely associated with handling or consumption of undercooked poultry meat products (Oosterom et al., 1984; Harris et al., 1986). Other known causative agents like untreated drinking water or raw milk are considered to be of minor importance in the Dutch situation (Health Council of The Netherlands, 1988). De Boer et al. (1991) reported a *Campylobacter* contamination rate of 41% in a total of 1880 fresh poultry products at the Dutch retail level. Bolder (1992) found a contamination rate of 63% in 190 poultry products of Dutch origin.
Contamination routes by which live poultry becomes colonized by *Campylobacter* are still under investigation and research is mainly focused on broiler production (Van de Giessen et al., 1992; Humphrey et al., 1993; Kapperud et al., 1993). The present study investigates the possible role of breeder flocks in the epidemiology of *Campylobacter* in poultry production. Dutch breeder farms were screened for the presence of *Campylobacter* spp. and *Salmonella* spp. Serotyping of *Campylobacter* isolates was used to identify epidemiological factors contributing to the *Campylobacter* infection of these breeder flocks.

Materials and methods

Sampling

A total of 43 separately housed broiler breeder flocks on nine different farms (Farms A to I) were screened for the presence of *Campylobacter* spp. and *Salmonella* spp. These nine farms belonged to one integrated company and all birds were of the same breed. Flock sizes ranged from 400 to 9,000 birds. Birds on one farm were always of the same age. Sampling took place in August and September 1990. Each flock was sampled by taking 30 fresh caecal droppings of individual birds. Samples were collected with sterile swabs and transported to the laboratory in modified Amies transport medium without charcoal (Probact transport swabs,

Technical Service Consultants Ltd., UK). Samples were analyzed within 48 hours of collection.

Campylobacter isolation and serotyping

Swabs were directly streaked on *Campylobacter* blood-free selective medium (CCD-agar, Oxoid CM 739), with cefoperazone (Oxoid SR 125, 32 mg/l medium) and actidione (Sigma, 100 mg/l medium) as selective agents. CCD-agar plates were incubated micro-aerobically in anaerobic jars with CampyPak Plus (BBL 71045). Incubation was at 37 °C for two days. Suspect colonies were examined under the microscope for the typical corkscrew shape and rapid, darting motility. A latex agglutination test (Meritec Campy, Meridian Diagnostics) was used for final confirmation.
When possible, one *Campylobacter* isolate per positive sample was serotyped by using the heat-stable serotyping system according to Penner (Penner and Hennessy, 1980) and as modified by Jacobs-Reitsma et al. (1994d). Sixty-five different sera were available. Serotypes are indicated by their O-numbers.

Salmonella isolation and serotyping

For each flock, five pooled samples of two swabs each were incubated at 37 °C in 9 ml buffered peptone water (BPW, Oxoid CM 509). After incubation for 18-24 hours, 0.1 ml BPW was inoculated into 10 ml Rappaport-Vassiliadis enrichment broth (RV, Oxoid CM 669). After 24 and 48 hours of incubation at 42 °C, a loopful of RV broth was streaked on brilliant green agar (BGA, Oxoid CM 329). The BGA plates were incubated 18 to 24 hours at 37 °C. Suspect colonies on BGA plates were confirmed as *Salmonella* spp. using polyvalent O-serum (National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands). One *Salmonella* isolate per flock was sent to the RIVM for serotyping.

Results

The results of *Campylobacter* and *Salmonella* sampling and serotyping are summarized in Table 3.1.

Table 3.1. *Isolation and serotyping results of* Salmonella *and* Campylobacter *sampling of the nine breeder farms under study (no* Campylobacter *serotyping results available for Farm C).*

Farm A, 40-week-old birds.

flock 1	flock 2	flock 3	flock 4	flock 5
S: neg [1]	S: neg	S: neg	S: neg	S: neg
C: neg [2]	C: neg	C: neg	C: neg	C: neg

Farm B, 6-week-old birds.

flock 1	flock 2	flock 3	flock 4
S: neg	S: neg	S: neg	S: neg
C: neg	C: neg	C: neg	C: neg

Farm C, 6-week-old birds.

flock 1	flock 2	flock 3	flock 4
S.typhim.[3]	*S.typhim.*	S: neg	S: neg
C: 7/30[4]	C: neg	C: 24/30	C: 11/30

Farm D, 6-week-old birds.

	flock 1
	S: neg
C types	C: 26/30
O37	1[5]
O48	8

Farm E, 20-week-old birds.

C types	flock 1 *S.infantis* C: 22/30	flock 2 *S.infantis* C: 4/20	flock 3 *S.infantis* C: 8/20	flock 4 S: neg C: 11/20	flock 5 *S.infantis* C: 10/20	flock 6 *S.infantis* C: 10/20	flock 7 S: neg C: 5/20
O28	4	-	3	1	2	2	1
O37	9	2	-	4	1	2	3
O53	-	-	1	-	-	-	-
O56	-	-	-	-	1	-	-
O59	-	-	-	-	1	-	-
nt[6]	4	1	1	4	4	2	1

Farm F, 40-week-old birds.

C types	flock 1 *S.infantis* C: 30/30	flock 2 *S. infantis* C: 30/30	flock 3 S: neg C: neg	flock 4 S: neg C: neg	flock 5 S: neg C: neg	flock 6 S: neg C: 30/30
O2	-	-	-	-	-	14
O5	16	15	-	-	-	-
O63	-	1	-	-	-	-
nt	1	1	-	-	-	1

(Continued)

Table 3.1. (Continued).

Farm G, 40-week-old birds.

C types	flock 1 S: neg C: 16/30	flock 2 S: neg C: 24/30	flock 3 S: neg C: 28/30	flock 4 S: neg C: 23/30	flock 5 S: neg C: 28/30	flock 6 S: neg C: 24/30	flock 7 S: neg C: 17/30
O2	5	2	7	1	2	3	-
O30	-	12	13	17	17	9	7
O31	-	-	-	1	-	-	-
O53	6	5	4	-	2	2	4
O63	3	-	-	-	-	-	-
nt	2	-	1	2	3	2	3

Farm H, 40-week-old birds.

C types	flock 1 *S.typhim.* C: 25/30	flock 2 S: neg C: 28/30
O1,44	-	4
O2	2	10
O21	1	3
O38,42	-	1
nt	-	5

Farm I, 20-week-old birds.

C types	flock 1 S: neg C: neg	flock 2 S: neg C: 5/25	flock 3 S: neg C: 8/30	flock 4 *S.typhim.* C: 7/30	flock 5 S: neg C: 9/30	flock 6 S: neg 12/30	flock 7 *S.typhim.* C: 16/30
O1,44	-	1	-	-	-	1	-
O2	-	1	-	1	-	1	2
O13	-	-	-	-	2	-	-
O24,54	-	-	1	3	6	2	6
O46	-	1	4	-	-	4	-
O47	-	2	-	-	-	-	-
O48	-	-	1	-	-	-	-
O57	-	-	1	-	1	3	4
O59	-	-	-	1	-	1	2
nt	-	-	1	2	-	1	1

[1] S neg : *Salmonella* not isolated
[2] C neg : *Campylobacter* not isolated
[3] S. typhim.: *S. typhimurium* isolated
[4] Number of *Campylobacter* positive samples/number of samples tested
[5] Number of *Campylobacter* isolates with the indicated serotype
[6] nt: non typable

Campylobacter was isolated from 29 out of the 43 flocks (67%) and *Salmonella* was isolated from 12 flocks (28%). Both *Campylobacter* and *Salmonella* bacteria were found in 11 flocks (26%); on the contrary, 13 flocks (30%) were negative for the two species.

Two out of the nine farms in this study, one with four houses of 6-week-old birds and one with five houses of 40-week-old birds, were both *Campylobacter*- and *Salmonella*-free at the time of sampling. Two other farms were *Salmonella*-free, but were *Campylobacter*-positive. Both *Campylobacter*-positive and -negative flocks on the same farm were found in three cases. The *Campylobacter* colonization rate within positive flocks ranged from 20 to 100%.

Swab samples from Farm E (flocks 1-6) had to be re-analyzed, due to technical problems, after an additional five days of storage at room temperature. The average isolation rate from these samples was 40% (range between 20 and 55%).

A total of 330 *Campylobacter* isolates were serotyped. Forty-three strains (13%) were non typable with the 65 sera used. A total of 19 different *Campylobacter* serotypes were isolated in this study. One similar pattern of serotypes was generally observed in all houses on a farm. Up to 10 different serotypes could be found on a single farm (Table 3.1).

Only two different *Salmonella* serotypes were isolated in this study. *S. infantis* was isolated on Farms E and F. *S. typhimurium* was isolated on Farms B, H and I.

Discussion

Breeder flocks generally are kept in production for about 45 weeks. Birds are often raised on a special rearing farm for the first (non-productive) part of their life. At about 18 weeks of age, birds are transferred to the breeding farm. This makes it more complicated to trace the origin of *Campylobacter* colonization of these flocks. Nevertheless, isolation and serotyping results in this study gave some useful indications of the *Campylobacter* infection pathways involved.

The 6-week-old birds from flocks 3 and 4 on Farm B originated from the same parents and hatch as the birds from flocks 3 and 4 on Farm C. *Campylobacter*, however, was detected only in the birds on Farm C. This makes the parent flock or hatchery an unlikely source of the *Campylobacter* infection on Farm C.

Flocks 6 and 7 on Farm G originated from the same parents, but from different

ones than flocks 2, 3, 4, 5 or the separate flock 1. All flocks were raised in one house with three different pens on rearing Farm X. At 18 weeks of age, the flocks were transported to seven different houses on Farm G. Despite the differences in origin of the birds, the *Campylobacter* serotypes O2, O30, and O53 were dominantly present over all seven flocks. This clearly points to a horizontal *Campylobacter* transmission rather than to a vertical one. Most likely the major *Campylobacter* contamination occurred at rearing Farm X. The two flocks of Farm H were raised on this rearing farm during the same period as Farm G flocks. Both farms shared the *Campylobacter* serotype O2 contamination.

Some flocks of Farm I were also raised on rearing Farm X, but after Farm G and H flocks had left. Flocks 1 and 2 were raised on Farm X for six weeks and then moved to the production Farm I. The same procedure was followed for half the number of birds in flocks 3 to 6. The other half of these flocks, as well as flock 7, were reared totally on Farm I. Serotyping results indicate that types O24,54, O57 and O59 might well be originating from the production farm itself, whereas serotypes O1,44, O2, and O46 more likely originate from the rearing farm. Serotype O1,44 was also found on Farm H, and serotype O2 on boths Farm H and G, from which all birds were raised for the first period of their lives on the particular Farm X. There is no explanation for the absence of *Campylobacter* in flock 1 on Farm I, however.

A positive correlation (chi-square analysis, $P < 0.05$) was found between *Campylobacter* and *Salmonella* colonization within breeder flocks. A similar correlation was found within broiler flocks at slaughter (Jacobs-Reitsma et al., 1994a), and might be another indication for farm-dependent factors in the contamination routes of these bacteria.

In general, the large variation in *Campylobacter* serotypes found on a single farm or even in a single house makes interpretation of serotyping results precarious and again indicates the complexity of *Campylobacter* epidemiology in poultry.

This study reveals that, like in broiler flocks, *Campylobacter* colonization in breeder flocks is quite common. *Campylobacter* colonization of breeder flocks implicates a potential role of vertical transmission via the egg to progeny. However, both this study and several other observations do not support a vertical transmission route until now. Laboratory experiments with various types of artificial infection of eggs did not easily result in contaminated day-old chicks (Doyle, 1984; Neill et al., 1985; Shanker et al., 1986). Also under practical conditions, Shanker et al. (1986) did not

find any evidence for vertical transmission from *Campylobacter* excreting breeder flocks to their progeny. In longitudinal studies on broiler farms, no *Campylobacter* was isolated from any of the hatchery samples examined and presence of *Campylobacter* in the broiler chicks could not be demonstrated during the first two weeks after hatching (Engvall et al., 1986; Jacobs-Reitsma et al., 1994e).

Breeder flocks have to be recognized as another reservoir of *Campylobacter*. However, it might be more appropriate to consider them as a potential risk factor in horizontal transmission routes in poultry production, rather than as a risk factor in vertical transmission via the egg.

CHAPTER 4

Isolation of *Campylobacter* from Eggs and Organs of Naturally Contaminated Laying Hens Housed in Battery Cages and Aviaries

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ABSTRACT Two groups of laying hens, housed in battery cages and aviaries, were screened for presence of *Campylobacter* during their productive life. Layers in both housing systems became colonized with *Campylobacter*, though contamination was delayed in battery-housed flocks. Isolation rates within positive flocks, however, were close to 100% and layers became permanent excreters of *Campylobacter* in their caecal droppings up to the time of slaughter. Penner serotyping of *Campylobacter* isolates indicated horizontal transmission from the environment. From 3 out of 179 eggs from *Campylobacter* excreting flocks, *Campylobacter* was isolated from the faecally contaminated shell surfaces. However, *Campylobacter* was not isolated from the yolks of these eggs nor from a further 40 yolks examined. At slaughter, *Campylobacter* was isolated from the caeca of 93 of the 100 birds tested. *Campylobacter* was also isolated from samples of ovaries (4x), magnum (7x), isthmus (11x), and uterus (14x), with a total of 21 birds having a degree of *Campylobacter* contamination in the reproductive tract. This contamination most likely originated from an external faecal contamination. Vertical transmission of the organism from *Campylobacter* excreting breeder flocks to their progeny via the egg is unlikely to occur. Also, the results obtained do not indicate that table eggs are a likely source of *Campylobacter* infection in humans. Nevertheless, the impact of *Campylobacter* contamination in the reproductive tract of laying hens has to be determined more closely.

Introduction

Campylobacter has been recognized as a major cause of human enteritis. Foods of animal origin may serve as vehicles of infection. In many reports, an association has been made between campylobacteriosis and consumption of raw or undercooked poultry meat (Griffith and Park, 1990; Skirrow, 1991).
Intestinal carriage of *Campylobacter* in broiler flocks at slaughter is frequently high (Jacobs-Reitsma et al., 1994a). Contamination during processing results in 40-100% of the retail poultry products to be contaminated with *Campylobacter* (Stern, 1992).
Laying hens also are reported to be carriers of *Campylobacter*, but the role of table eggs or laying hens in the transmission of *Campylobacter* has been less well studied than *Campylobacter* epidemiology in broiler production (Shane, 1992).
The present study was undertaken to elucidate *Campylobacter* epidemiology in laying hens. Laying hens in both battery cages and aviaries were tested for presence of *Campylobacter* throughout their life. At slaughter, *Campylobacter* contamination of internal organs, especially the reproductive tract, was studied. Also egg shells and yolks were examined for presence of the organism. Heat-stable serotyping of isolated *Campylobacter* strains was used as epidemiological marker (Penner and Hennessy, 1980).

Materials and methods

Laying hen flocks

Two separate groups (A and B) of laying hens were studied. Group A consisted of four subgroups: 3500 Isabrown Warren hens in three-tier battery cages (flock A1), 3500 Lohman LSL hens in battery cages (flock A2), 3500 hens of both breeds on a Tiered Wired Floor system (TWF) (Ehlhardt et al., 1989) (flocks A3 and A4, respectively).
At the rearing farm (Farm S), flocks A1 and A2 (battery cages) were housed in one building. Flocks A3 and A4 (TWF system) were housed in another building in two rooms separated by a feeding room. At the production farm (Farm O), flocks A1 and A2 were housed in one room and flocks A3 and A4 were housed in another

room in the same building, though the latter were physically separated by a fence. The flocks were followed from day-of-hatch up to (late) slaughter at 89 weeks of age during the period from June 1991 to February 1993.
Group B consisted of a total of 3500 Isabrown Warren hens divided into four subgroups (flocks B1 to B4). Flocks B1 and B2 were housed on a deep litter system in floor pens and flocks B3 and B4 were housed in battery cages (Van Niekerk and Reuvekamp, 1994). The flocks on these two different housing systems were raised on two different rearing farms (Farms X and Y). On the production Farm O, Flocks B1 to B4 were housed in four adjacent units within one building.
The flocks were followed from the day they arrived on the egg production farm until slaughter at 40 weeks of age during the period from October 1993 to May 1994.

Campylobacter analysis

Flocks were tested for presence of *Campylobacter* by collecting fresh caecal droppings with sterile swabs. Ten swabs were taken per flock and per sampling time. Environmental samples on the rearing Farm S, consisting of fresh faecal samples from a breeder flock, cattle, geese, and fancy poultry, were taken twice during the rearing period when the laying hens were seven and twelve weeks of age. Sample analysis was performed within one hour after sample collection.
Swab samples of group A were streaked on *Campylobacter* blood-free selective medium (CCDA, Oxoid CM 739), with cefoperazone (Oxoid SR 125, 32 mg/l medium) and cycloheximide (Sigma, 100 mg/l medium) as selective agents. Cefoperazone and amphotericin-B (Oxoid SR 155E, 32 and 10 mg/l medium) were used as selective agents in CCDA plates for group B swab samples. The CCDA plates were incubated micro-aerobically in anaerobic jars with CampyPak Plus (BBL 71045) for two days at 37 °C. Suspect colonies exhibiting *Campylobacter* morphology were examined under the microscope for typical corkscrew shape and rapid, darting motility. A latex agglutination test (Group A: Meritec Campy, Meridian Diagnostics; Group B: Microscreen M46, Mercia Diagnostics) was used for final confirmation.
Serotyping of *Campylobacter* isolates was performed according to the modifications described by Jacobs-Reitsma et al. (1994d) of the Penner serotyping scheme (Penner and Hennessy, 1980).

Sampling of internal organs

A total of 100 laying hens, equally divided among flocks A1, A3, B1, and B3, were killed with CO_2 gas and internal organs were aseptically excised and examined for the presence of *Campylobacter*. The external surface of the organ under investigation was disinfected with 70 % (v/v) ethanol. A sterile scalpel was used to make an incision in the organ and the internal surface/material of the organ was sampled with a sterile swab. Swabs were directly placed into 10 ml *Campylobacter* selective enrichment broth (CCDB, Bolton et al., 1984, with the selective agents as described for the CCDA plates) and incubated micro-aerobically for 24 hours at 42 °C. A loopful of material from the enrichment tube was streaked on a CCDA plate and microaerobically incubated for 48 hours at 42 °C. Suspect colonies were handled as described for the flock samples.

The following organs were tested in all 100 birds: liver, both caeca, ovaries (in fact the largest yellow follicle), magnum, isthmus, and uterus. Twenty birds were additionally tested for presence of *Campylobacter* in: crop, glandular stomach, muscular stomach (gizzard), duodenum, ileum and colon.

Samples from both caeca were directly streaked on CCDA plates before the two swabs were placed into one tube of enrichment broth.

A minimum of five isolates from each of the *Campylobacter*-positive organs of one laying hen from flock A3 were serotyped. Isolates from the reproductive tract of flocks B1 and B3 hens were also serotyped.

Sampling of eggs

Fresh eggs were collected within 24 hours of being laid. A total of 140 eggs were obtained from group A one week before slaughter. Twenty of these eggs had faecally contaminated shells, but were free of cracks or other damage. A total of 119 eggs, all with faecally contaminated shells but without damage, were obtained from group B three days before slaughter.

No differentiation was made between eggs from the different flocks within group A or B.

Individual eggs were placed in a Stomacher bag with 50 ml of CCDB broth. Eggs were carefully rubbed for one minute and placed at 37 °C for four hours to resuscitate damaged *Campylobacter* cells (Humphrey, 1989). Subsequently they

were placed at 42 °C for another 20 hours and a loopful of enrichment broth was streaked on CCDA plates. CCDA plates were examined for suspect colonies after micro-aerobic incubation for 48 hours at 42 °C.

Egg yolk was examined for presence of *Campylobacter* by breaking the egg carefully and placing the intact yolk on a egg yolk separator. The yolk surface was wiped with a clean tissue and sterilized with 70% (v/v) ethanol. A sterile scalpel was used to make an incision and yolk material was taken out with a sterile swab. The swab samples were handled as described for the organ testing and included the resuscitation step as described for the shell samples.

Statistical analysis

The significance of differences between groups was assessed by chi-square analysis. Differences between groups were considered to be significant at $P < 0.05$.

Results

Flock sampling

Campylobacter was isolated from flocks A3 and A4 (TWF system) at six weeks of age, with 10 out of 10 samples being positive in both flocks. The flocks remained *Campylobacter*-colonized up to slaughter at 89 weeks.

Campylobacter was not isolated from flocks A1 or A2 before 16 weeks of age. At this time, just before transport to the production farm, four out of 25 swab samples were found to be *Campylobacter*-positive in flock A1. Flock A2 was found to be colonized by *Campylobacter* at 18 weeks (on the production farm).

Campylobacter isolation percentages varied between 60 and 100%. The variation can be explained by the difficulty in collecting the right (fresh) caecal sampling material.

Serotyping results of the isolates from laying hen flocks A1 to A4, as well as from the environmental samples on Farm S, are presented in Table 4.1, and are subdivided into the period on the rearing farm and that on the production farm.

Flocks B1 and B2 (deep litter system) were *Campylobacter*-positive when they arrived on the production Farm O, whereas no *Campylobacter* was isolated from

flocks B3 and B4 (battery system) at this time (17 weeks of age). *Campylobacter* was first isolated from flock B3 at 30 weeks of age and from flock B4 at 34 weeks of age. Isolation rates within all flocks usually yielded 9 or 10 out of the 10 samples *Campylobacter*-positive.

Table 4.1. *Serotype distribution of* Campylobacter *isolates from flocks A1 and A2 (battery) and A3 and A4 (TWF) at the rearing and production farms.*

	Serotype														
	1	2	3	5	13	16	24	30	37	42	43	53	65	nt	Total
Rearing:															
Environment*	2#	-	-	5	1	-	-	6	2	2	-	-	-	2	20
A1 and A2	-	-	-	-	-	-	-	-	-	-	-	9	-	2	11
A3 and A4	-	14	-	-	13	2	-	-	-	-	1	16	1	31	78
Production:															
A1 and A2	20	1	1	1	3	-	-	6	-	-	-	-	-	36	68
A3 and A4	4	4	6	-	9	1	12	4	-	-	-	-	3	19	62
organs 1 hen (A3)	-	-	13	-	-	-	12	10	5	-	-	-	-	8	48
Total	26	19	20	6	26	3	24	26	7	2	1	25	4	98	287

nt: non typable

* *Campylobacter* isolates from a young breeder flock, geese, and fancy poultry, also present on the rearing farm.

\# Number of isolates.

Sampling of internal organs

Campylobacter colonization did not affect performance of the hens during their laying cycle and no pathological abnormalities were observed when the birds were slaughtered. *Campylobacter* isolation results of internal organ sampling of the 100 laying hens are summarized in Table 4.2. The pattern of *Campylobacter* isolation from the reproductive tract samples of individual birds is summarized in Table 4.3.

Table 4.2. Campylobacter *isolation from internal organs of laying hens from flocks A1, A3, B3, and B1.*

	A1 TWF	A3 battery	subtotal	B3 TWF	B1 battery	subtotal	Total
Liver	2/25*	4/25	6/50	0/25	0/25	0/50	6/100
Crop	2/ 5	3/ 5	5/10	3/ 5	3/ 5	6/10	11/ 20
Glandular stomach	1/ 5	2/ 5	3/10	3/ 5	3/ 5	6/10	9/ 20
Muscular stomach	0/ 5	1/ 5	1/10	2/ 5	0/ 5	2/10	3/ 20
Duodenum	0/ 5	1/ 5	1/10	3/ 5	3/ 5	6/10	7/ 20
Ileum	1/ 5	2/ 5	3/10	4/ 5	4/ 5	8/10	11/ 20
Colon	4/ 5	5/ 5	9/10	4/ 5	5/ 5	9/10	18/ 20
Caeca	22/25	25/25	47/50	22/25	24/25	46/50	93/100
Ovary	0/25	2/25	2/50	0/25	2/25	2/50	4/100
Magnum	0/25	1/25	1/50	0/25	6/25	6/50	7/100
Isthmus	1/25	4/25	5/50	1/25	5/25	6/50	11/100
Uterus	3/25	4/25	7/50	2/25	5/25	7/50	14/100

* Number of samples *Campylobacter*-positive/number of samples tested

Table 4.3. *Pattern of* Campylobacter *isolation from the caeca and reproductive tract samples of 100 individual birds.*

	Campylobacter isolation pattern									
Caeca	-	+	+	+	+	+	+	+	+	+
Ovary	-	-	-	-	-	-	+	-	-	+
Magnum	-	-	-	-	+	-	-	+	+	+
Isthmus	-	-	-	+	-	+	-	+	+	-
Uterus	-	-	+	-	-	+	+	-	+	+
Number of birds	7	72	4	4	2	4	2	1	2	2

\- : no *Campylobacter* isolated
\+ : *Campylobacter* isolated

Caecal samples were considered positive if at least one of the two caeca yielded *Campylobacter* after direct plating, or if *Campylobacter* was isolated after enrichment of the caecal samples. Ninety-two out of 100 caecal samples tested were *Campylobacter*-positive after direct plating, whereas only 80 samples yielded *Campylobacter* after the selective enrichment procedure ($P < 0.05$).

No significant differences were found between the hens from the floor-housed system and the battery system within both groups A and B, except for the magnum isolates between flock B1 and B3 ($P < 0.01$). The most remarkable difference between total results of group A and group B was the total absence of *Campylobacter* isolates from the livers in group B and the 6 contaminated livers out of the 50 tested in group A ($P < 0.025$).

On one occasion only, *Campylobacter* was not isolated from the caeca of *Campylobacter*-positive birds. *Campylobacter* colonization of parts of the digestive tract was not correlated with colonization of parts of the reproductive tract.

Serotyping results of isolates of one bird from flock A3 and serotyping results from isolates from flocks B1 and B3 are presented in Tables 4.4 and 4.5, respectively.

Table 4.4. *Serotyping results of* Campylobacter *isolates from internal organs from one laying hen from flock A3.*

	Serotype					
	3	24,54	30	37	nt	Total
Liver	-	-	-	5[#]	-	5
Crop	-	1	3	-	1	5
Duodenum	2	2	-	-	1	5
Jejunum	4	-	2	-	-	6
Ileum	1	-	4	-	-	5
Caecum	5	-	1	-	-	6
Ovary	1	3	-	-	1	5
Magnum	-	3	-	-	2	5
Uterus	-	3	-	-	3	6
Total	13	12	10	5	8	48

nt: non typable
[#] Number of isolates.

Table 4.5. *Serotyping results of* Campylobacter *isolates from caeca and reproductive tract samples from flocks B1 and B3.*

	Serotype				
	1,44	3	37	nt	Total
Caecum	4[#]	3	-	6	13
Ovary	-	-	-	1	1
Magnum	1	-	-	1	2
Isthmus	1	1	-	4	6
Uterus	1	1	2	2	6
Total	7	5	2	14	28

nt: non typable
[#] Number of isolates.

Egg sampling

No *Campylobacter* was isolated from the yolk or shell of 20 eggs with dirty shells, nor from shells from 40 eggs or the yolk from 80 eggs of standard consumer quality produced by group A birds.
From three out of 119 eggs from group B hens, *Campylobacter* was isolated from the faecally contaminated shell surfaces, but not from any of the yolks.

Discussion

Both birds in batteries (flocks A1 and A2) and those in the aviary (A3 and A4) were raised on the same rearing farm at the same time, so husbandry and environmental conditions were identical. *Campylobacter* was isolated from the floor-housed hens when they were six weeks of age. *Campylobacter* in the battery-housed flocks was first detected only at 17 or 19 weeks of age. The battery system could not prevent the layers from *Campylobacter* contamination, but at least delayed the transmission to the birds.
Floor-housed flocks B1 and B2 were already *Campylobacter*-positive when they arrived at the production Farm O, but it took at least another 12 weeks before *Campylobacter* was isolated from the battery-housed flocks 3 and 4. Here also, the *Campylobacter* contamination in flocks B1 and B2 did not immediately lead to *Campylobacter* transmission to the adjacent flocks B3 and B4. This might have been due to the influence of the housing system, though hygienic precautions might also have been quite effective.
As was observed in broiler flocks (Engvall et al., 1986; Jacobs-Reitsma et al., 1994e), the spread of *Campylobacter* throughout the floor-housed birds was rapid. Most likely this is favoured by the opportunity for the birds to consume faecal material and contaminated litter or water.
However, isolation rates within a positive flock in battery cages were also close to 100% within two weeks after first isolation, although conditions in cages do not seem to be as favourable for spreading the organism as floor pens. *Campylobacter* transmission via aerosols might play a role here (Evans, 1992).
Invariably, high isolation rates were observed in the *Campylobacter*-colonized

flocks, indicating that the hens in both housing systems were permanently excreting the organism in their caecal droppings. Doyle (1984) tested 148 laying hens over a 42-week period and found only 8% of the birds to be permanent excreters of the organism, with a maximum isolation rate of 25%. In contrast to our study, these hens were all individually caged.

A wide variety of *Campylobacter* serotypes were isolated from the laying hens (Group A). The diversity increased with time, indicating a constant flow of new campylobacters entering the flocks. Serotypes O3 and O24,54 were not isolated during the rearing period and most likely originated from a contamination source on the production farm. All other serotypes were already isolated at the rearing farm and were brought to the production farm with the laying hens at 17 weeks. Similar serotype distribution among the battery flocks and the floor-housed flocks indicate an interchange of serotypes, so the flocks themselves have to be regarded as contamination sources for each other.

Campylobacter was isolated from three out of the 179 egg shells tested. This contamination was related to eggs carrying faecal matter, and thus not being of consumer quality. Doyle (1984) found two out of 226 egg shells from a *Campylobacter*-excreting laying hen flock to be contaminated with *Campylobacter*. One of the two eggs had a dirty shell.

Campylobacter-excreting laying hens may produce externally contaminated eggs. Relative to the high percentage of laying hens that were excreting the organism, only very few egg shells were found to be contaminated. This can be explained by the fact that survival of *Campylobacter* on the egg shell will be very poor, because of the sensitivity of *Campylobacter* to drying (Doyle, 1984). Studies on egg shells artificially contaminated with *Campylobacter*-positive faecal material showed that *Campylobacter* could not be detected after storage at room temperature for more than 48 hours (Shane, 1986; Jacobs-Reitsma, 1992d).

No *Campylobacter* was isolated from any of the 219 egg yolks tested. Similarly, Doyle (1984) did not isolate *Campylobacter* from any of the contents of 226 eggs. *Campylobacter* infections in humans via table eggs is not considered to be very likely under practical circumstances.

Both groups A and B laying hens gave quite similar results with respect to *Campylobacter* contamination of intestinal and reproductive tract samples (Table 4.2).

Campylobacter was isolated from 93 out of the 100 laying hens examined at slaughter. Direct plating of caecal material was significantly more sensitive than when only the selective enrichment procedure was used.

Campylobacter isolation from the different parts of the intestinal tract were not surprising, because this part is regarded to be the main side of colonization of *Campylobacter* in the commensal relationship with the poultry host (Shane, 1992).

More surprising was the isolation of *Campylobacter* from organs from the reproductive tract. To the author's knowledge, the only report on examination of reproductive tract samples for *Campylobacter* contamination was on experimentally infected laying Japanese quails. Maruyama and Katsube (1990) isolated *Campylobacter* from uterus (2x), magnum/isthmus (2x) and yellow follicles (1x) from three out of the seven hens studied. They suggested the contamination of the yellow follicles to originate from invasion via the blood or lymph.

In the current study however, the increasing isolation rate from ovaries to uterus parts (Table 4.2), as well as the pattern of *Campylobacter* isolation within individual birds (Table 4.3), indicate an external contamination with faecal material and the highly motile *Campylobacter* ascending the reproductive tract. Serotypes isolated from the reproductive tract organs did not differ from those isolated from intestinal tract materials (Tables 4.4 and 4.5), which may also indicate the role of external contamination of the reproductive tract via faeces.

Vertical transmission of *Campylobacter* from *Campylobacter*-excreting breeder flocks to their progeny via the egg is unlikely to occur. Also, it may be concluded that under normal circumstances the consumption of table eggs is unlikely to lead to *Campylobacter* infection in humans. Nevertheless, the impact of *Campylobacter* contamination in the reproductive tract of laying hens has to be determined more closely.

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CHAPTER 5

Epidemiology of *Campylobacter* spp. at Two Dutch Broiler Farms

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ABSTRACT Broiler flocks on two Dutch poultry farms were screened weekly for the presence of *Campylobacter* in fresh caecal droppings during eight consecutive production cycles. Hatchery and fresh litter samples were taken at the start of each new cycle. Water, feed, insects, and faeces of domestic animals present on the farms were also included in the sampling. Penner serotyping of isolates was used to identify epidemiological factors that contribute to *Campylobacter* colonization in the broiler flocks. Generally, broiler flocks became colonized with *Campylobacter* at about three to four weeks of age with isolation percentages of 100%, and stayed colonized up to slaughter. A similar pattern of serotypes was found within the various broiler houses on one farm during one production cycle. New flocks generally showed also a new pattern of serotypes. Most serotypes isolated from the laying hens, pigs, sheep, and cattle were different from those isolated from the broilers at the same time. *Campylobacter* serotypes from darkling beetles inside the broiler houses were identical to the ones isolated from the broilers. No *Campylobacter* was isolated from any of the hatchery, water, feed or fresh litter samples. Conclusive evidence of transmission routes was not found, but results certainly point towards horizontal transmission from the environment. Horizontal transmission from one broiler flock to the next one via a persistent contamination within the broiler house, as well as vertical transmission from breeder flocks via the hatchery to progeny, did not seem to be very likely.

Introduction

Live poultry is often found to be colonized by *Campylobacter*. The birds are healthy carriers of this bacterium, which may be found at counts of 10^6 - 10^9 colony forming units per gram of faeces (Altmeyer et al., 1985; Mead and Hinton, 1989). *Campylobacter* colonization of live poultry may affect public health in two ways: 1) direct effect of the organism causing disease in workers at farms or processing plants (Jones and Robinson, 1981; Christenson et al., 1983), and 2) contamination of consumer-ready poultry products, which in turn may cause food-borne illness (Blaser et al., 1983; Oosterom et al., 1983; Skirrow, 1991).

Intervention strategies have to be developed in order to reduce contamination rates and thus the risk of *Campylobacter* infections in humans. Possible means of preventing *Campylobacter* colonization of live broiler flocks have to be found in the first place. Unfortunately, epidemiology of *Campylobacter* colonization in poultry is not yet fully understood. Feed, water, domestic animals, insects, rodents, and wild birds have all been suggested as possible sources of horizontal transmission (Mead and Hinton, 1989; Evans, 1992; Shane, 1992). Vertical transmission via the egg, from *Campylobacter*-positive breeder flocks to their progeny, has not been found to be very likely up to now (Shanker et al., 1986; Annan-Prah and Janc, 1988; Van de Giessen et al., 1992).

A longitudinal study on the presence of *Campylobacter* in broiler flocks and environmental sources was carried out at two Dutch poultry farms. *Campylobacter* isolates were serotyped (Penner and Hennessy, 1980; Jacobs-Reitsma et al., 1994d) in order to identify epidemiological factors contributing to the *Campylobacter* colonization of broiler flocks on these farms.

Materials and methods

Farms

In a preliminary study on three Dutch broiler farms (Farms A, B, and C), all broiler flocks had been *Campylobacter*-free during two consecutive production periods (Jacobs-Reitsma, 1990).

Presence of *Campylobacter* on two other broiler farms (Farms D and E) was monitored from November 1989 until January 1991, during eight consecutive broiler production cycles. One cycle is the rearing period of broilers during about six weeks in a separate chicken house on one farm.
Both farms used fresh straw for litter material on concrete floors. After each cycle all used litter was removed and the houses, including the drinkers and feeding system were cleaned and disinfected. The broiler houses remained empty for at least one week before new flocks arrived.
Separate boots and clothing, as well as a boot disinfection bath were present in each house on both farms. However, proper use of these facilities was sometimes doubted.
Both farms operated a system in which a proportion of the flock was removed from the houses at about five weeks of age, with the remaining birds being slaughtered at about six weeks.

Four broiler houses were present on Farm D. House D1 and D2 were completely separate buildings, but houses D3 and D4 were connected by a joint feeding room. All broiler flocks were of the same breed and were obtained from a single hatchery. A letter-code system was used to indicate the different parent flocks involved and the data are listed in Table 5.2. Flock sizes ranged from 20,000 birds in houses D3 and D4 to 30,000 birds in houses D1 and D2.
Chicks received a combined spray vaccination against Newcastle disease (NCD) and infectious bronchitis (IB) at the hatchery, and a vaccination via the drinking water against Gumboro disease at 12 days of age.
The birds were fed pelleted feed: a starter feed for the first 17 days after hatching, a grower feed for the next three weeks, and a finisher feed during the last five to seven days before slaughter. Furazolidone was added to the starter feed at a concentration of 200 mg/kg for the first seven days of life to prevent *E. coli* infections. The coccidiostats nicarbazin (125 mg/kg) and salinomycin (60 mg/kg) were added to the starter feed and the grower feed, respectively. Avoparcine was added to all types of feed as a growth promoter at a concentration of 10 mg/kg.
Chicks received tap water; with bell-type drinkers being used in houses D1, D3 and D4, and a nipple system in house D2.
Rodents (mainly mice) and insects (flies and darkling beetles) were controlled with appropriate chemicals.

On a separate location 0.5 km away, this farmer managed a flock of 9,000 laying hens.

Three separate broiler houses (houses E1 to E3) were present on Farm E. Broilers were always of the same breed and were obtained from a single hatchery. The letter-coded data on the different parent flocks involved are listed in Table 5.3.
A total of 30,000 chicks was placed in house E1 and after about two weeks, half the number of birds was moved to house E2. Broiler flocks in house E3 consisted of about 30,000 birds.
Vaccination was carried out as described for Farm D, except that the combined NCD/IB vaccination took place at the farm.
The birds were fed pelleted feed, but no detailed information on the types of feed was available. Chicks received tap water, with bell-type drinkers being used in houses E1 and E2, and a nipple system in house E3.
In addition to the broilers, pigs, cattle (milking cows) and sheep were present on this farm.
Rodents and insects were controlled by mechanical means (however, these were unsuccessful at least for darkling beetles and lesser mealworms in house 1 and 3).

Sampling

Broiler flocks on Farms D and E were screened for the presence of *Campylobacter* spp. during eight consecutive cycles.
Hatchery debris and fluff, and paper pads from the transport coops for the day-old chicks, as well as fresh litter were sampled at the start of each new production cycle.
Ten to 30 samples of fresh caecal droppings were taken weekly from each of the broiler houses. Samples were collected with sterile swabs. When *Campylobacter* was not detected on the farm, 30 caeca from the broiler flock were taken at slaughter for examination.
Environmental samples examined for the presence of *Campylobacter* included tap water, feed, insects and faeces of domestic animals present on the farms.
Water and feed samples were taken during the first or second week of each flock cycle and supplementary water samples were taken on Farm E during the fifth week

of each cycle in the different houses. Water and feed samples were taken from the separate storage bins, before coming in contact with the broilers.
Darkling beetles and lesser mealworms (*Alphitobius diaperinus* and larvae) were collected every time they were observed on the floor or walls of a broiler house.
Laying hens were examined by taking individual swab samples of fresh caecal droppings. Pigs, sheep and cattle were sampled by taking pools of fresh faecal material.
Examination of samples for the presence of *Campylobacter* spp. was carried out within two hours after sample collection.

Isolation and serotyping of *Campylobacter* spp.

Swab samples were directly streaked on *Campylobacter* blood-free selective medium (CCD-agar, Oxoid CM 739), with cefoperazone (Oxoid SR 125, 32 mg/l medium) and actidione (Sigma, 100 mg/l medium) as selective agents. CCD-agar plates were incubated micro-aerobically in anaerobic jars with CampyPak Plus (BBL 71045). Incubation was at 37 °C for two days. Suspect colonies were examined under the microscope for the typical corkscrew shape and rapid, darting motility. A latex agglutination test (Meritec Campy, Meridian Diagnostics) was used for final confirmation.
Hatchery, fresh litter, water and feed samples were diluted 1:9 (w/v) in the selective enrichment medium CCD-broth (per litre: 25 g nutrient broth no. 2 [Oxoid CM 67], 4 g bacteriological charcoal [Oxoid L9], 3 g casein hydrolysate [Oxoid L41], 1 g sodium deoxycholate [Merck 6504], 0.25 g ferrous sulphate [Merck 3965], 0.25 g sodium pyruvate [Merck 6619] and the selective agents cefoperazone and actidione as described for the CCD-agar). Water and feed were tested in portions of 25 ml and 25 g, respectively.
The external surface of the collected insects was disinfected with 70% ethanol, and one sample of 10-50 insects was ground and transferred into 10 ml of CCDB. One gram of faecal samples of pigs, sheep and cattle was brought into 10 ml of CCDB.
The CCD-broth was incubated microaerobically for two days at 42 °C. A loopful of broth was then subcultured on a CCD-agar plate and handled as described for the swab samples.

Over 800 *Campylobacter* isolates from both broilers and environmental sources were serotyped by using the heat-stable serotyping system according to Penner (Penner and Hennessy, 1980) with the modifications described by Jacobs-Reitsma et al. (1994d).

Results

Screening of broiler farms for presence of *Campylobacter* spp.

Figures 5.1 and 5.2 summarize the results of *Campylobacter* isolations from the broilers during eight consecutive cycles on Farms D and E, respectively. As shown in Figure 5.1, broiler flocks in houses D3 and D4 arrived on the farm one week before the flocks in houses D1 and D2. Broiler flocks in house E3 arrived three to five days after the flocks in houses E1 and E2.

Campylobacter was isolated from 18 of the 32 flocks examined (56%) on Farm D. On Farm E, 20 of the 22 flocks studied (91%) were found to be colonized by *Campylobacter*.

The earliest detection of *Campylobacter* was in cycle 4 on Farm E, when the broilers were 13 days old. Only one out of the 30 samples was *Campylobacter*-positive at that time, but the isolation rate was already 100% (30/30) seven days later.

Generally, the broilers became colonized by *Campylobacter* at about three to four weeks of age. Once *Campylobacter* had entered a flock, all broilers became colonized within one week and remained colonized up to slaughter with isolation rates close to 100%.

The results of *Campylobacter* isolations from environmental samples are summarized in Table 5.1. No *Campylobacter* was isolated from any of the hatchery or paper pad samples. *Campylobacter* was also not isolated from any of the fresh litter, water and feed samples examined in this study.

The laying hens on Farm D were found to be colonized by *Campylobacter* throughout the whole survey. The same was true for the pigs on Farm E. However, *Campylobacter* was isolated less frequently from the sheep on this farm, and only isolated twice from the milking cows (both times during cycle 8). *Campylobacter* isolations from the domestic animals were independent of the presence of *Campylobacter* in the broiler flocks.

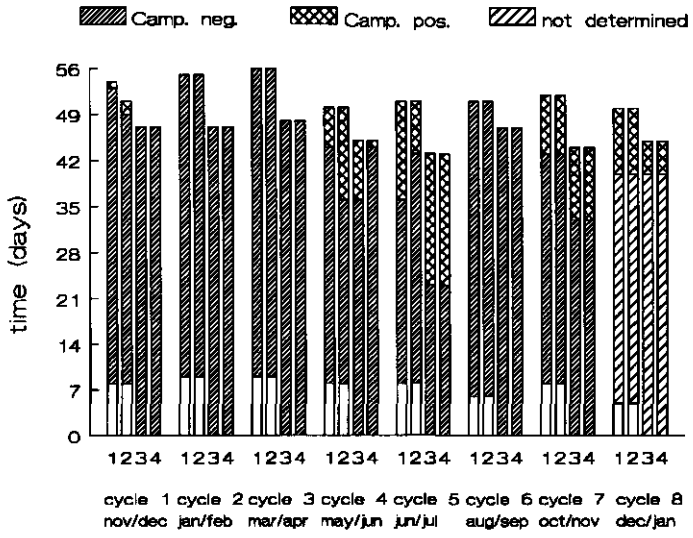

Figure 5.1. *Results of* Campylobacter *screening of broiler flocks in houses D1 to D4 (November 1989 to January 1991).*

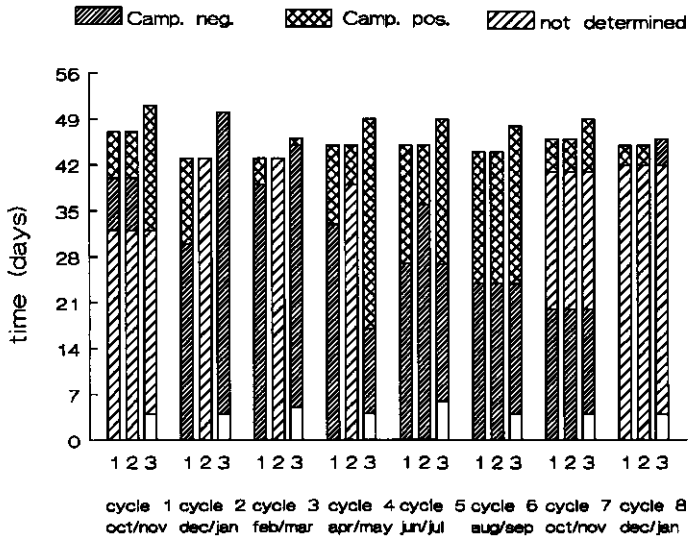

Figure 5.2 *Results of* Campylobacter *screening of broiler flocks in houses E1 to E3 (October 1989 to January 1991).*

No darkling beetles or lesser mealworms were observed in house E2, but in houses E1 and E3 they were found during all cycles. Especially during summer (cycle 4-6) their presence was abundant, but generally they could not be captured before the second or third week of a production cycle, as they were still hiding in the roofs. *Campylobacter* species were isolated from these insects on several occasions, but never before the broilers in that particular house were found to be colonized by *Campylobacter*.

Serotyping of *Campylobacter* isolates

A total of 809 *Campylobacter* isolates from both broilers and environmental samples were serotyped; 14% of these isolates were not typable with the 65 sera available. Serotyping results of the *Campylobacter* isolates from broilers and laying hens on Farm D and Farm E are presented in Table 5.2 and Table 5.3, respectively.

Table 5.1. Isolation of Campylobacter *from environmental samples of Farms D and E.*

Type of sample	*Campylobacter* isolations
Hatchery samples (total)	0/187*
Paper pads (total)	0/115
Fresh litter (total)	0/48
Water (total)	0/80
Feed (total)	0/56
Laying hens (Farm D)	71/82
Pigs (Farm E)	40/45
Sheep (Farm E)	10/17
Cattle (Farm E)	12/26
Insects (Farm E, house 1 and 3)	36/104

* Number of *Campylobacter*-positive samples/number of samples tested

Table 5.2. Serotyping results of Campylobacter *strains isolated on farm D.*

				Penner serotype																		
	P	H	C	1	2	3	13	14	16	24	28	30	37	49	51	53	56	59	60	65	nt	Σ
Cy 1	m	1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	2	-	-	3
	b	2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30	-	-	30
	m	3	-																			-
	f,k	4	-																			-
		lh	+	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	1	-	2	4
Cy 2	f,n	1	-																			-
	f,m	2	-																			-
	r	3	-																			-
	r	4	-																			-
		lh	x																			
Cy 3	l	1	-																			-
	l,p	2	-																			-
	l	3	-																			-
	l	4	-																			-
		lh	+	2	1	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	4
Cy 4	g	1	+	-	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10
	f,s	2	+	-	33	-	-	-	-	4	4	-	-	-	-	-	-	-	-	-	1	42
	s	3	+	-	9	-	-	-	-	1	1	-	-	6	-	-	-	-	-	-	-	17
	f,j	4	+	-	3	-	-	-	-	1	1	-	-	6	-	-	-	-	-	-	-	11
		lh	x																			
Cy 5	j	1	+	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-	-	-	-	5
	h,s	2	+	-	-	1	1	-	-	-	-	-	-	6	-	-	-	-	-	-	21	29
	s	3	+	-	-	-	1	-	-	-	-	-	10	-	-	-	-	3	-	-	-	14
	f,s	4	+	-	-	-	-	-	-	-	-	-	8	-	-	-	-	-	-	-	1	9
		lh	+	18	-	-	-	-	2	-	-	-	-	-	-	1	-	5	1	1	-	28
Cy 6	d	1	-																			-
	s	2	-																			-
	s	3	-																			-
	c,q	4	-																			-
		lh	x																			
Cy 7	j,s	1	+	-	-	-	-	1	-	-	-	-	-	-	-	-	13	-	-	-	-	14
	k,o,	2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	34	-	-	-	4	38
	s	3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	17	-	-	-	-	17
	i	4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	19	-	-	-	-	19
	i,l	lh	+	2	-	-	-	8	3	4	2	-	-	-	1	1	-	2	-	-	5	28
Cy 8	i,m	1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	10	-	-	-	-	10
	a,m,	2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	10	-	-	-	-	10
	o	3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	10	-	-	-	-	10
	e	4	+	-	-	-	-	-	-	-	-	-	-	-	-	2	9	-	-	-	-	11
	i	lh	x																			

Cy n = Cycle n (n = 1 to 8) H = House: number; lh = laying hens
P = Parent flock code C = *Campylobacter*: + = positive; - = negative; x = not tested
nt = non typable Σ = Total

Table 5.3. Serotyping results of Campylobacter *strains isolated on farm E.*

				Penner serotype																			
	P	H	C	1	2	5	6	11	13	16	30	33	34	37	46	48	53	57	59	65	o	nt	Σ
Cy 1		1 b	+	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1
		1 d	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
		2 b	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
		3 b	+	-	-	-	1	-	-	2	-	-	-	-	-	-	-	-	-	1	-	-	4
		3 d	x																				-
		p	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1
		s	x																				-
		c	-																				-
Cy 2		1 b	+	-	-	-	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	26	29
		1 d	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	6
		2 b	x																				-
		3 b	-																				-
		3 d	-																				-
		p	+	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1
		s	-																				-
		c	-																				-
Cy 3		1 b	+	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	27	-	-	-	29
		1 d	-																				-
		2 b	x																				-
		3 b	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	-	-	-	10
		3 d	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	8	11
		p	+	-	-	-	1	-	-	-	-	-	-	-	1	-	-	-	-	-	1	-	3
		s	+	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
		c	-																				-
Cy 4		1 b	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	32	-	-	-	32
		1 d	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	2
		2 b	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12	-	-	1	13
		3 b	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11	-	-	-	11
		3 d	-																				-
		p	+	-	-	2	-	-	-	-	-	-	-	-	-	1	-	-	1	-	1	-	5
		s	x																				-
		c	-																				-
Cy 5	v	1 b	+	-	17	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	21
		1 d	+	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
	v	2 b	+	-	4	-	-	-	-	-	-	-	-	26	-	-	-	-	-	-	-	-	30
	x	3 b	+	-	-	-	-	-	-	-	-	-	-	15	-	-	-	-	-	-	-	-	15
		3 d	-																				-
		p	+	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	1	-	3
		s	-																				-
		c	x																				-

(Continued)

Table 5.3. (Continued)

				Penner serotype																			
	P	H	*C*	1	2	5	6	11	13	16	30	33	34	37	46	48	53	57	59	65	o	nt	Σ
Cy 6	z	1 b	+	-	-	-	-	-	-	-	-	-	-	20	11	-	-	-	6	-	-	7	44
		1 d	+	-	-	-	-	-	-	-	1	-	-	1	-	-	-	-	1	-	-	-	3
	z	2 b	+	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	10	-	-	2	14
	z,α	3 b	+	-	-	-	-	-	-	-	9	-	-	1	1	-	-	-	1	-	-	2	14
		3 d	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	2
		p	+	-	-	-	2	-	-	-	1	-	-	-	-	-	-	-	-	-	1	-	4
		s	+	-	-	3	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1	-	5
		c	x																				-
Cy 7	u,y,δ	1 b	+	-	-	-	-	8	-	-	-	-	2	-	-	-	-	1	5	-	-	13	29
		1 d	+	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	3	-	-	2	6
	u,y,δ	2 b	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	-	-	-	15
	x	3 b	+	-	-	-	-	-	-	-	-	-	-	-	9	-	-	-	9	-	-	-	18
		3 d	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	-	-	-	6
		p	x																				-
		s	x																				-
		c	x																				-
Cy 8	w	1 b	+	2	-	-	-	1	-	-	-	-	-	-	-	-	5	-	-	-	-	1	9
		1 d	+	1	-	-	-	-	-	-	-	-	-	-	-	-	2	-	2	-	-	-	5
	w	2 b	+	4	-	-	-	4	-	-	-	-	-	-	-	-	-	-	3	-	-	1	12
	ß	3 b	-																				-
		3 d	-																				-
		p	+	-	-	2	-	-	-	-	4	-	-	-	-	-	-	-	-	-	8	1	15
		s	x																				-
		c	+	-	5	-	-	-	1	-	-	-	-	-	-	-	-	-	3	-	-	3	12

Cy n = Cycle n (n = 1 to 8)
P = Parent flock code
H = House: number & b = broilers, d = darkling beetles; p = pigs; s = sheep; c = cattle
C = *Campylobacter*: + = positive; - = negative; x = not tested
o = Other Penner Serotypes: 20, 23, 24, 39 or 56 (only pigs *or* sheep)
nt = non typable
Σ = Total

On Farm D, 15 different serotypes were isolated from the various broiler flocks, with a maximum of four different serotypes within one flock at one sampling time. Generally, a similar pattern of serotypes was found in the four separate broiler houses within one production cycle. Consecutive cycles, however, generally showed a different pattern of serotypes. A total of 13 different serotypes was isolated from the laying hens on the farm, and up to nine different types were detected at one sampling time. Some serotypes (e.g., O1,44; O24,54; O60) were found at different sampling times, whereas other types (e.g., O30, O2; O65; O51) were detected only once during the whole survey. Some serotypes were isolated from both the broilers and the laying hens on this farm, but not always at the same sampling time. Some serotypes frequently isolated from the broilers (like O49; O37; O56) were not isolated at all from the laying hens.

Fifteen different serotypes were isolated from the various broiler flocks on Farm E, and up to five different serotypes could be isolated from one flock at the same time. Serotype O59 was the most frequently isolated serotype from the broiler flocks on Farm E and was found during cycles 3, 4, 6, 7, and 8. Apart from the frequent isolation of this particular serotype, the pattern of serotypes found during the various cycles was quite variable. The serotype pattern in all three broiler houses within one production cycle was much more similar, although even within one cycle a change in serotype pattern was sometimes observed.

Serotypes isolated from the darkling beetles and lesser mealworms were also present in the broilers during the same cycle and mostly in the house from where the insects were collected.

Ten different serotypes were observed in the 32 pig isolates that were serotyped. Only three of them were also found in the broilers, but O59 (cycle 4) and O30 (cycle 6) were isolated at the same sampling time from both pigs and broilers.

Due to the low isolation rate from sheep and cattle on Farm E, only a small number of isolates from these animals were serotyped. Only O59 was isolated from both cattle and broilers at the same sampling time (cycle 8).

Discussion

Campylobacter species were frequently isolated from the broiler flocks. *Campylobacter* was generally isolated at the earliest between three and four weeks of age. Campylobacters were never isolated before the birds were two weeks of age.

The relatively late detection of *Campylobacter* in cycle 1 on Farm D and cycle 3 on Farm E may be caused by the practice of partial unloading at five weeks of age. The transport crates or the loading people may bring in a *Campylobacter* contamination and leave the remaining part of the flock enough time to become colonized in the last week before slaughter.

Once *Campylobacter* was isolated from a flock, all broilers in that particular broiler house proved to be colonized within one week and isolation rates in the flocks remained at 100% up to the time of slaughter. Similar findings, both on first detection time and isolation rates within flocks, were also reported in other longitudinal studies (Altmeyer et al., 1985; Pokamunski et al., 1986; Engvall et al., 1986).

However, not all broiler flocks were found to be *Campylobacter*-positive at the end of the production cycle. Moreover, both *Campylobacter*-positive and -negative flocks could be present at the same time on one farm, even up to slaughter. Therefore, *Campylobacter*-free rearing of broiler flocks can be achieved and is certainly an important tool in the prevention of human campylobacteriosis via poultry meat products.

No *Campylobacter* species were detected in any of the hatchery samples (Table 5.1). Broilers from identical parent flocks were found to be *Campylobacter*-colonized in one production cycle and *Campylobacter*-free in another. These data do not support the likelihood of vertical transmission as an important pathway; although Dutch breeder flocks are frequently found to be *Campylobacter* carriers (Jacobs-Reitsma et al., 1994f). Other studies report similar findings with respect to vertical transmission (Shanker et al., 1986; Annan-Prah and Janc, 1988; Van de Giessen et al., 1992).

Since *Campylobacter* was not isolated from any of the fresh litter, water and feed samples, these factors did not seem to be of major importance for transmission of *Campylobacter* in this study.

The majority of *Campylobacter*-positive broiler flocks were colonized with more than one serotype at the same time, as was observed in other studies on broiler flocks as well (Pokamunski et al., 1986; Sjögren and Kayser, 1989). The serotype distribution within a flock sometimes changed during the production cycle (data not shown). Most likely, this reflects the constant flow of other campylobacters entering a broiler house, which may result in a serotype dominance of a newly introduced

strain. Laboratory experiments with broilers challenged with two different *Campylobacter* serotype strains showed a complete dominance of one type over the other within one week (Jacobs-Reitsma, 1992c). Results on repeated serotyping of several *Campylobacter* strains did not suggest any serotype instability within the strains as a possible cause (Jacobs-Reitsma et al., 1994d).

Similar *Campylobacter* serotype patterns were found at the same time in different flocks (from different breeder flocks) on one farm. This clearly indicates transmission from the same environmental sources or cross-contamination from adjacent houses. Consecutive cycles generally showed a different serotype pattern, so carry over of *Campylobacter* within a house from the previous flock did not seem to occur on these farms.

The laying hens on Farm D were found to be permanent carriers of a number of *Campylobacter* serotypes and should therefore be regarded as a potential source of *Campylobacter* contamination for broiler flocks on this farm.

The laying hens might occasionally have been the source of the *Campylobacter* contaminations (cycle 5, serotype O59 or cycle 7, serotype O14). Most other serotypes, frequently found in the broilers, were not isolated from the layers at any time. Other, undefined, sources are therefore suspected to play an important role in transmission of *Campylobacter*.

In accordance with other studies (Van de Giessen et al., 1992; Weijtens et al., 1993), pigs (Farm E) were found to be permanent carriers of a variety of *Campylobacter* serotypes. However, a similar serotype in both pigs and broilers during one cycle was detected only twice (cycle 4: O59 and cycle 6: O30).

No clear correlation, except for the O59 isolates in cycle 8, was found between serotypes from sheep and cattle and serotypes from broilers on Farm E. But this might partly be due to the relatively low number of sheep and cattle isolates that could be tested. Nevertheless, these ruminants, as well as pigs and laying hens, cannot be excluded as potential sources of *Campylobacter* infection for broilers on a farm.

In contrast to the findings of Jones (1992), *Campylobacter* was isolated on several occasions from the internal contents of darkling beetles and lesser mealworms, although this never occurred before the organism was also isolated from the broilers. Identical serotypes were isolated from both the insects and broilers within broiler houses E1 and E3. This might indicate an infection route from insects to

broilers, but the reverse infection route from broilers to insects is just as likely. More detailed studies are needed to determine the survival and colonization potential of *Campylobacter* in these insects under the less optimal conditions of an empty (and generally cold) broiler house.

Conclusions

The results from this study, and in particular the large number of different *Campylobacter* serotypes isolated from both broiler flocks and environmental sources, indicate the complexity of *Campylobacter* epidemiology in broiler flocks.
Conclusive evidence for certain transmission routes was not found, but the results certainly point towards horizontal transmission from the environment as a major one.
Neither a horizontal transmission from one broiler flock to the next one due to a persistent contamination within the broiler house, nor a vertical transmission from breeder flocks via the hatchery to their progeny, seem to be very likely.
Intervention procedures against horizontal transmission have to be studied further and the effectiveness of strict hygienic practices during the whole production period, such as was described as being successful in small scale experiments by Van de Giessen et al. (1992) and Humphrey et al. (1993), has to be evaluated on a larger scale.

CHAPTER 6

Caecal Carriage of *Campylobacter* and *Salmonella* in Dutch Broiler Flocks at Slaughter: A One-Year Study

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ABSTRACT From March 1992 to March 1993, 187 Dutch broiler flocks were screened to assess their *Campylobacter* and *Salmonella* carriage. Every four weeks at least 10 flocks, at three different slaughterhouses, were screened for presence of these bacteria. Twenty-five caecal samples were taken from each flock. *Campylobacter* spp. were isolated from 153 out of 187 broiler flocks (82%). *Campylobacter* carriage of flocks showed seasonal variation, with the highest contamination rate (100%) during the period June to September and the lowest (50%) in March. *Salmonella* carriage of the flocks did not show a distinct seasonal variation. *Salmonella* spp. were isolated from 49 out of 181 broiler flocks (27%). A positive correlation was found between *Campylobacter* and *Salmonella* colonization within flocks. Data on farming conditions and husbandry practices were studied to identify possible risk factors for *Campylobacter* and *Salmonella* colonization of Dutch broiler flocks.

Introduction

Campylobacter bacteria are known to be an important cause of human diarrhoea. In The Netherlands, *Campylobacter* is isolated from patients with acute gastroenteritis in more than 10% of cases; for *Salmonella* this incidence is about 5%. Campylobacteriosis is mainly a foodborne infection, and products of animal origin, especially poultry products, play an important role in transmission (Blaser et al., 1983; Health Council of The Netherlands, 1988).
Results from a Dutch sentinel study revealed a peak in (human) *Campylobacter* isolations during summer (Hoogenboom-Verdegaal et al., 1990). Studies in the U.K. (Skirrow, 1987) and Norway (Kapperud and Aasen, 1992) also reported a summer peak in human cases of campylobacteriosis. This was suggested to be related to a peak in animal *Campylobacter* colonization (Jones et al., 1990).
A one-year study was carried out to assess *Campylobacter* carriage in Dutch broiler flocks at slaughter and to investigate a possible seasonal variation in presence of this organism. Detection of *Salmonella* was also included in the study. General data on the sampled flocks, with respect to age and broiler line, as well as more specific data on husbandry practices on broiler farms associated with one particular slaughterhouse, were studied to identify possible risk factors for *Campylobacter* and *Salmonella* colonization of Dutch broiler flocks.

Materials and methods

Sample collection

Dutch broiler flocks were screened to assess the *Campylobacter* and *Salmonella* carriage during the period from March 1992 to March 1993. At least 10 flocks were sampled for the presence of these bacteria every four weeks. Broiler flock sizes ranged from 3,000 to 40,000 birds. Data on broiler line (mainly Ross or Cobb), broiler age (varying from 31 to 48 days, majority = 42 days), and geographical location of the broiler farm (mainly in the regions "north", "central-east", and "south") were recorded for all sampled broiler flocks. Samples were mainly taken at three different slaughterhouses. Each broiler flock was sampled by collecting 25

caeca at the slaughterline just after mechanical evisceration. Caeca were placed into sterile plastic bags and stored at 4 °C. Sample analysis usually was within six hours after sample collection.

A total of 4675 caecal samples, representing 187 broiler flocks from 160 different farms, were examined for presence of *Campylobacter*. One hundred and eighty-one flocks were also examined on presence of *Salmonella* (1448 pooled samples).
The 25 caeca per broiler flock were all taken from different broilers, and only one of the two caeca per bird was examined. Additionally, the second caecum of five broilers per flock was also examined for presence of *Campylobacter* for a total of 162 flocks, resulting in 810 extra samples.
Meteorological data on temperature and relative humidity, collected at Meteorologisch Waarnemingsstation Haarweg (Wageningen, The Netherlands), are shown in Figure 6.1. These data are considered to be an indication of weather in The Netherlands during the sampling period.

Campylobacter analysis

Caeca were aseptically opened and caecal content was sampled with sterile cotton swabs. Swabs were streaked on *Campylobacter* blood-free selective medium (CCD-agar, Oxoid, CM 739), with cefoperazone (Oxoid, SR 125, 32 mg/l medium) and cycloheximide (Sigma, 100 mg/l medium) as selective agents. From October 1992 on cefoperazone and amphotericin-B (Oxoid, SR 155E, 32 and 10 mg/l medium) were used as selective agents in CCD-agar plates, without any notable changes in performance. The CCD-agar plates were incubated under micro-aerobic conditions (CampyPak Plus, BBL 71045). After two days of incubation at 37 °C, suspect colonies were microscopically examined for typical corkscrew shape and rapid, darting motility. A latex agglutination test (Microscreen M46, Mercia Diagnostics Ltd.) was used for final confirmation. A limited number of isolates was also tested for hippurate hydrolysis using the method described by Hwang and Ederer (1975). A flock was considered positive for *Campylobacter*, when *Campylobacter* bacteria were detected in at least one of the 25 caecal samples per flock.

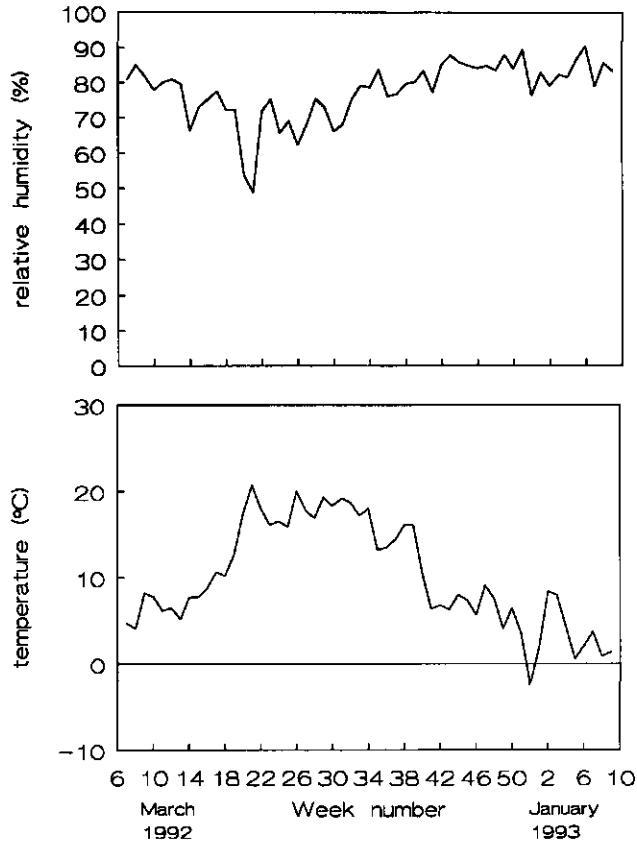

Figure 6.1. Relative humidity and temperature during the period February 1992 to March 1993; Meteorologisch Waarnemingsstation Haarweg, Wageningen, The Netherlands.

Salmonella analysis

Per flock, eight pooled samples of three swabs each were incubated at 37 °C for 18 to 24 hours in Buffered Peptone Water (BPW, Oxoid, CM 509). Three drops of BPW were inoculated onto the surface of a modified semi-solid Rappaport-Vassiliadis plate (MSRV, Oxoid, CM 910) and 0.1 ml BPW was inoculated into 10

ml Rappaport-Vassiliadis enrichment broth (RV, CM 669). The MSRV plates were incubated for 18 to 24 hours at 41.5 °C and examined for opaque halos of growth. After 24 and 48 hours of incubation at 41.5 °C, RV tubes were streaked on Brilliant Green Agar (BGA, Oxoid, CM 329). The BGA plates were incubated 18 to 24 hours at 37 °C. Material from the outside edge of the halo on MSRV plates and suspect colonies on BGA plates were subcultured on BGA plates and confirmed to be *Salmonella* spp. with a latex agglutination test (Oxoid, FT 203). A flock was considered to be positive for *Salmonella*, when *Salmonella* bacteria were detected in at least one of the eight pooled caecal samples.

Statistical analysis

Logistic regression analysis was carried out with the statistical computer program Genstat (Genstat 5 Committee, 1987). The significance of differences between groups was assessed on basis of accumulated analysis of deviance, using the chi-square test. Differences between groups were considered to be significant at $P < 0.05$.

Results

Campylobacter and *Salmonella* colonization of flocks

Campylobacter spp. were isolated from 153 out of 187 broiler flocks (82%). A *Campylobacter* isolation rate of ≥ 80% was found in 116 out of the 153 *Campylobacter*-positive flocks.

For 810 broilers, both caeca were examined for presence of *Campylobacter*. Identical results for both caeca were found in 98% of these cases. In 16 pairs (from 13 different flocks) one caecum was *Campylobacter*-positive, and the other caecum was *Campylobacter*-negative. The number of positive samples per 25 broilers within these 13 flocks was generally low.

A total of 268 *Campylobacter* isolates, originating from 70 flocks, were tested on their ability to hydrolyse hippurate. One hundred and seventy-seven isolates (66%) were hippurate-positive (*C. jejuni*) and the other 91 isolates were hippurate-negative.

Salmonella spp. were isolated from 49 out of 181 broiler flocks (27%). *Salmonella*-positive flocks generally yielded only one or two positive samples out of the eight pooled samples.

Both *Campylobacter* and *Salmonella* bacteria were found in 45 flocks (25%); on the contrary, 30 flocks (17%) were negative for the two species.
Figure 6.2 gives the percentages of *Campylobacter*- and *Salmonella*-positive broiler flocks per sampling week. The figure shows a seasonal pattern in *Campylobacter* contamination of broiler flocks, with the highest contamination rate (100%) during the period from June to September (Weeks 26 to 38) and the lowest (50%) in March (Week 10). *Salmonella* contamination of the flocks does not show a distinct seasonal pattern.

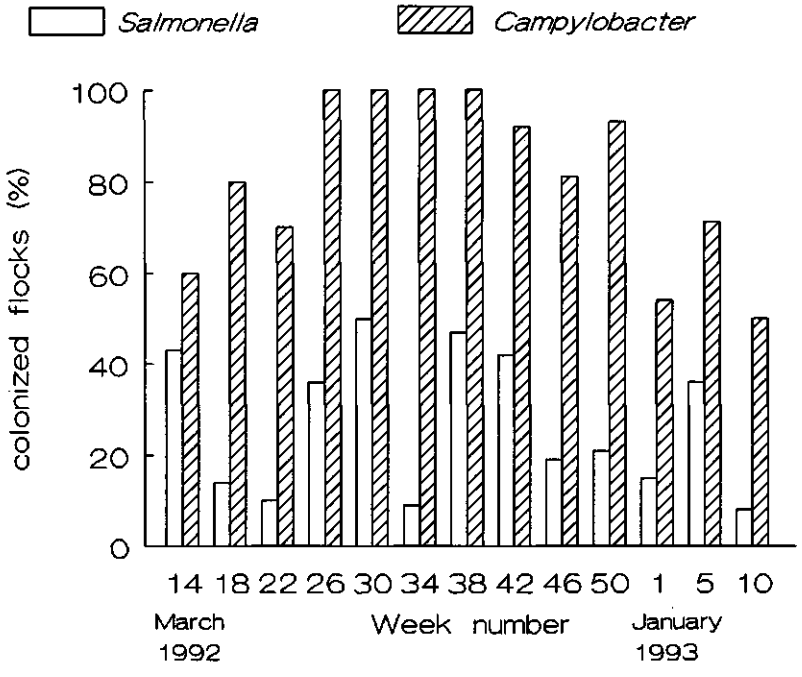

Figure 6.2. Percentage of Salmonella *and* Campylobacter *colonized broiler flocks from March 1992 to March 1993.*

Statistical analysis of possible risk factors

In the 27 cases in which two flocks from the same farm were sampled, only the flock sampled first was used in statistical analysis. Logistic regression analysis revealed that presence of *Campylobacter* in broiler flocks showed a seasonal effect ($P < 0.005$), whereas presence of *Salmonella* in broiler flocks did not. Broiler line, broiler age at slaughter, and geographical location of the broiler farm had no significant influence on the presence of *Campylobacter* and *Salmonella* in broiler flocks. The slaughterhouse, however, was significantly associated with presence of *Campylobacter* in broiler flocks. At Slaughterhouse D, a lower contamination rate was found than at Slaughterhouses A and C ($P < 0.05$).
A positive correlation ($P < 0.05$) 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.*

One of the slaughterhouses provided data on farming conditions and husbandry practices of their farmers in the study. Statistical analysis on the separate factors (Table 6.1) was carried out to investigate possible associations with presence of *Campylobacter* or *Salmonella* in broiler flocks. The broiler line was associated with presence of *Campylobacter* in the broiler flocks under study ($P < 0.01$). The geographical location of the broiler farm, the type of drinking water system, and the absence of dogs were all associated with presence of *Salmonella* in the broiler flocks ($P < 0.05$). A strong association was observed between the use of special feed (without ingredients of animal origin) and the absence of *Salmonella* in the flocks ($P < 0.005$).

Discussion

The present study clearly indicates a high caecal carriage rate of *Campylobacter* (82%) and a lower carriage rate of *Salmonella* (27%) in Dutch broiler flocks at slaughter. In contrast to *Salmonella*, *Campylobacter* isolation rates within positive flocks were generally high (mean = 81%). Other studies report Campylobacter

Table 6.1. *Potential risk factors in* Campylobacter *and* Salmonella *colonization of broiler flocks. Data on farmers, related to one particular slaughterhouse.*

Factor	Type	*Campylobacter*	(%)		*Salmonella*	(%)	
Region	North	12/13[1]	92		7/13	54	
	Mid-east	33/35	94		4/33	12	*
	South	24/27	89		6/26	23	
Line	Ross	32/32	100	**	9/31	29	
	Cobb	31/37	84		8/36	22	
Hatchery	X	44/50	88		9/48	19	
	Y	16/16	100		6/16	38	
Litter	Straw	8/8	100		3/7	43	
	Wood shavings	22/26	85		7/26	27	
Feed supplier	K	46/50	92		12/48	25	
	L	21/23	91		5/22	23	
Special feed	Yes	12/14	86		0/14	0	***
	No	66/72	92		23/69	33	
Feeding system	Chain	14/15	93		2/15	13	
	Pan	34/36	94		8/34	24	
	Mixed	5/7	71		3/7	43	
Water source	Public service	48/53	91		12/51	24	
	Well	6/6	100		2/6	33	
Water system	Nipples	13/16	81		3/14	21	
	Cups	14/15	93		6/15	40	*
	Bells	17/18	94		1/18	6	
	Mixed	8/8	100		4/8	50	
Hygiene score	Good	14/17	82		5/15	33	
	Standard	32/34	94		8/34	24	
	Below standard	8/8	100		1/8	13	
Pigs present	Yes	11/13	85		3/13	23	
	No	43/46	93		11/44	25	
Cattle present	Yes	14/15	93		3/15	20	
	No	40/44	91		11/42	26	
Dog(s) present	Yes	31/35	89		5/34	15	*
	No	22/23	96		9/22	41	
Other poultry	Yes	4/4	100		2/4	50	
	No	49/54	91		12/52	23	

1 No. of colonized flocks/no. of flocks tested.
* Difference between or among factor groups ($P < 0.05$).
** Difference between factor groups ($P < 0.01$).
*** Difference between factor groups ($P < 0.005$).

carriage rates in broiler flocks ranging from 18% of 176 flocks in Norway (Kapperud et al., 1993) to 76% of 49 flocks in the U.K. (Humphrey et al., 1993). Prescott and Gellner (1984) examined 60 Canadian broiler flocks and isolated *Campylobacter* spp. from 28 flocks (47%) and *Salmonella* spp. from 11 flocks (18%). Isolation rates within these positive flocks were 92 and 12% for *Campylobacter* and *Salmonella*, respectively. Broiler intestinal material, often containing *Campylobacter* or *Salmonella* bacteria or both, can easily contaminate large numbers of broiler carcasses during processing. If handled improperly, these contaminated end-products might lead to human illness. Prevention of colonization of broilers by these pathogens might thus have a considerable contribution to public health.

Seasonal variation in presence of *Campylobacter* and *Salmonella* and possible risk factors for broiler colonization were studied to obtain a better understanding of *Campylobacter* and *Salmonella* epidemiology in broiler flocks. Seasonal variation in presence of *Campylobacter* in broiler flocks, with a peak during the summer, was reported by Annan-Prah and Janc (1988). Kapperud et al. (1993) examined 176 broiler flocks at slaughter between April 1990 and April 1991 and 63% of the *Campylobacter*-positive flocks was detected during the period from August to November. However, Humphrey et al. (1993) did not find an apparent seasonal variation in *Campylobacter* presence in 49 flocks at slaughter between June 1990 and July 1991. The seasonal variation in presence of *Campylobacter* in broiler flocks in this study might be one of the explanations for the summertime peak found in human cases of campylobacteriosis in The Netherlands as described by Hoogenboom-Verdegaal et al. (1990).

The meteorological data on temperature (Figure 6.1) show some relation with the presence of *Campylobacter* in broiler flocks. Elevated temperatures coincide with high *Campylobacter* isolation rates. This might be a direct correlation with *Campylobacter* contamination itself, but also one (or more) sources of *Campylobacter* contamination might be temperature dependent (e.g., migratory birds, rodents, or darkling beetles). The variation in relative humidity (Figure 6.1) did not seem to be a direct explanation for the seasonal variation observed in this study.

A relatively lower *Campylobacter* isolation rate in broiler flocks was found in one of the three slaughterhouses in this study. It is not very likely that this difference

was due to the slaughtering process, because sampling was on caeca and not on end-products. Probably, factors related to the slaughterhouse or to integration, like selection of the broiler farms or involvement in hygiene programs, play a major role. A positive correlation was found between the presence of *Campylobacter* and the presence of *Salmonella* within broiler flocks. This correlation could be an indication for farm-dependent factors in *Campylobacter* and *Salmonella* contamination routes.

Kapperud et al. (1993) found the use of untreated well water, which is a common practice in Norway, to be an important risk factor in *Campylobacter* colonization of broiler flocks. Pearson et al. (1993) identified the drinking water from a borehole on a broiler farm to be the source of a persistent presence of *Campylobacter*. In our study, only six farms (10%) were using untreated well water instead of tap water. All six farms harboured *Campylobacter*-positive broiler flocks (too low a number to reach statistical significance, however).

Presence of *Salmonella* in the broiler flocks under study was strongly associated with the type of feed. All 14 flocks (100%) receiving a special type of feed were *Salmonella*-free, compared with 67% of 69 flocks receiving the 'normal' feed. Although no effect on *Campylobacter* colonization was found, this aspect certainly requires more detailed investigations.

In contrast to the analysis on the total group of farmers, broiler line was associated with presence of *Campylobacter* in the selected group of farmers (Table 6.1). Indications for differences in susceptibility towards *Campylobacter* colonization in different broiler lines were also reported by Bolder and Mulder (1991) and Stern et al. (1990).

Sixty-six percent of 268 *Campylobacter* isolates appeared to be *C. jejuni*. The remaining 34% of isolates were most likely to be *Campylobacter coli* or perhaps *Campylobacter lari*, because of the isolation on CCD-agar (Bolton et al., 1984). Other Dutch investigators report 28% (Endtz et al., 1991) or 14% (Bänffer, 1985) of *C. coli* isolates from poultry. Porcine isolates, however, were found to be *C. coli* for 98% (Bänffer, 1985). *C. coli* isolation from broiler flocks might therefore indicate a relation with *Campylobacter* infection of pigs. Kapperud et al. (1993) found pig breeding to be significantly associated with presence of *Campylobacter* in broiler flocks. In our study, 22% of the broiler farmers (Table 6.1) were also keeping pigs on their farm. However, no significant association was found between keeping pigs and presence of *Campylobacter* in the broiler flocks.

Factors like "presence of a hygiene barrier," "use of separate boots and clothing for each animal house," and "disinfection of boots before entering a broiler house" were used to assess hygiene practices on the farms. An increasing percentage of *Campylobacter*-positive flocks was found with a decreasing hygiene score. No such association was found for presence of *Salmonella* in broiler flocks. Future research will focus on improvement of hygienic measures on the farm level during rearing.

Acknowledgements

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CHAPTER 7

In Vitro Susceptibility of *Campylobacter* and *Salmonella* Isolates from Broilers to Quinolones, Ampicillin, Tetracycline, and Erythromycin.

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ABSTRACT Recently, an increased resistance of *Campylobacter* to fluoroquinolones, a newer class of antimicrobial agents in both human and veterinary medicine, has been reported. *Campylobacter* isolates (617) from 150 broiler flocks were tested for their susceptibility to cephalothin (control), ampicillin, tetracycline, erythromycin, and the quinolones nalidixic acid, flumequine, enrofloxacin, and ciprofloxacin by a disc diffusion method. Almost complete cross-resistance was found between the quinolones tested. *Campylobacter* isolates (181, 29%), originating from 55 flocks (37%), were quinolone resistant. *Salmonella* isolates (94) from 40 flocks were also tested for their antimicrobial susceptibility. Eight isolates (8.5%), from three broiler flocks (7.5%), showed resistance to nalidixic acid and flumequine (and tetracycline), but not to ciprofloxacin or enrofloxacin.

Introduction

Campylobacter and *Salmonella* are known to be important causes of human illness. From a Dutch sentinel study, it was estimated that *Campylobacter* and *Salmonella* cause respectively about 25,000 and 12,000 cases of foodborne disease per million (Dutch) people each year, respectively. Case control studies indicate poultry products as an important source of acute enteritis (Health Council of The Netherlands, 1988; Notermans and Hoogenboom-Verdegaal, 1992).

Fluoroquinolones are a newer class of antimicrobial agents, chemically related to nalidixic acid. In The Netherlands enrofloxacin and ciprofloxacin were introduced into veterinary and human therapy in 1987 and 1988, respectively. In 1991, Endtz et al. (1991) reported on the quinolone resistance of *Campylobacter* strains isolated in The Netherlands between 1982 and 1989 from human stools and poultry products. The prevalence of resistant strains isolated from poultry products increased over these years from 0% to 14% and the prevalence in human isolates increased from 0% to 11%. The emergence of quinolone resistant *Campylobacter* as well as *Salmonella* isolates was also reported for other European countries (Rautelin et al., 1991; Reina et al., 1992; Wray et al., 1992). The rapid increase in quinolone resistant bacteria may have implications for the treatment and prophylaxis of human diarrhoeal disease.

From March 1992 up to March 1993, 187 Dutch broiler flocks were screened to assess their intestinal *Campylobacter* and *Salmonella* status. *Campylobacter* spp. were isolated from 153 out of 187 broiler flocks (82%) and *Salmonella* spp. were isolated from 49 out of 181 broiler flocks (27%) (Jacobs-Reitsma et al., 1993). An additional study was undertaken to investigate the *in vitro* susceptibility of *Campylobacter* and *Salmonella* isolates from these broiler flocks to eight antimicrobial agents, especially to the (fluoro)quinolones nalidixic acid, flumequine, ciprofloxacin, and enrofloxacin.

Materials and methods

Campylobacter and *Salmonella* sampling

Between March 1992 and March 1993, 187 broiler flocks from 160 different farms were screened to assess their contamination with *Campylobacter* and *Salmonella*.

Campylobacter and *Salmonella* isolation was as described before (Jacobs-Reitsma et al., 1993). In short, *Campylobacter* spp. were isolated on modified *Campylobacter* blood-free selective medium (Oxoid CM 739), with cefoperazone (Oxoid SR 125) and actidione (Sigma, 100 mg/l medium) as selective agents. Material from the selective plates was subcultured on blood agar plates (nr. 2, Oxoid CM 271) supplemented with 5% sheep blood to obtain pure, single colonies. All *Campylobacter* incubations were under micro-aerobic conditions (CampyPak Plus, BBL 71045). *Campylobacter* isolates, 268 from 70 broiler flocks, were tested for hippurate hydrolysis, using the method described by Hwang and Ederer (1975).
Salmonella spp. were isolated after pre-enrichment in buffered peptone water (BPW, Oxoid CM 509) and selective enrichment in Rappaport-Vassiliadis broth (Oxoid CM 669) on brilliant green agar (BGA, Oxoid CM 329), or after pre-enrichment in BPW on modified semi-solid Rappaport-Vassiliadis plates (Oxoid CM 910). Material was subcultured on BGA to obtain pure and single colonies.

Antimicrobial susceptibility testing

Single colonies from the subcultures were used to test antimicrobial susceptibility. Where possible, five *Campylobacter* and *Salmonella* isolates per positive broiler flock were tested. Antimicrobial susceptibility was tested by the disc diffusion method (Bauer et al., 1966), as recommended by the National Committee for Clinical Laboratory Standards (1990). The following discs were used: cephalothin 30 µg (Oxoid, KF30), nalidixic acid 30 µg (Oxoid NA30), flumequine 30 µg (Oxoid, UB30), ciprofloxacin 5 µg (Oxoid, CIP5), enrofloxacin 5 µg (Bayer, ENR5), ampicillin 25 µg (Oxoid, AMP25), tetracycline 30 µg (Oxoid, TE30), and erythromycin 15 µg (Oxoid E15). Four discs per plate were used. The inoculum size was approximately 10^8 colony-forming units per plate.
Campylobacter strains were tested on blood agar plates. Plates were incubated micro-aerobically at 37 °C. After two days, inhibition zone diameters were measured with callipers. *Salmonella* strains were tested on Mueller-Hinton agar (Oxoid CM 337). After incubation for 24 hours at 37 °C, inhibition zone diameters were measured. Interpretation of *Salmonella* inhibition zones was according to the NCCLS (1990). *Campylobacter* isolates with an inhibition zone diameter ≤ 15 mm (including the disc diameter of 6 mm) were regarded as resistant (Endtz et al.,

1991). For enrofloxacin this criterion was ≤ 17 mm (both *Campylobacter* and *Salmonella* isolates, according to the manufacturers' instructions). Isolates with intermediate zones of resistance were considered to be susceptible. *E. coli* (ATCC 25922) and *St. aureus* (ATCC 25923) were used as control microorganisms.

Statistical analysis

The significance of differences between hippurate-positive and hippurate-negative *Campylobacter* isolates and the frequency of resistance was determined by using the chi-square test.

Results

Campylobacter isolates

The antimicrobial susceptibility of 617 *Campylobacter* isolates, originating from 150 broiler flocks, was determined. The numbers and percentages of resistant *Campylobacter* isolates per antimicrobial agent are listed in Table 7.1. All isolates were resistant to cephalothin (control). Almost complete cross-resistance was found between the quinolones tested. Twelve isolates were resistant to nalidixic acid only, and not to the other quinolones. Out of the 617 isolates, 181 (29.3%) were cross-resistant to the four quinolones tested. These isolates originated from 57 out of the 150 broiler flocks studied (38%). Additionally, 21 isolates (from six different flocks) were resistant to nalidixic acid, flumequine, and ciprofloxacin, but were only "intermediate resistant" to enrofloxacin, with an inhibition zone diameter of 18-25 mm.

Quinolone resistance in *Campylobacter* isolates did not coincide with higher frequencies of resistance to ampicillin, tetracycline, or erythromycin. Resistance to more than one agent, considering the quinolones to be one group, was found in 150 isolates (24.3%), and seven of these isolates were resistant to all antimicrobials tested.

Out of 268 *Campylobacter* isolates, 177 (66%) were hippurate-positive (*C. jejuni*) and 91 (34%) were hippurate-negative. The antimicrobial susceptibility of these isolates is listed in Table 7.2.

Table 7.1. Antimicrobial susceptibility of 617 Campylobacter *isolates.*

Antimicrobial agent	Disc content	Resistant *Campylobacter* isolates	
		number	percentage
Nalidixic acid	30 μg	214	34.7%
Flumequine	30 μg	202	32.7%
Ciprofloxacin	5 μg	202	32.7%
Enrofloxacin	5 μg	181	29.3%
Ampicillin	25 μg	218	35.3%
Tetracycline	30 μg	99	16.0%
Erythromycin	15 μg	26	4.2%
Cephalothin (control)	30 μg	617	100.0%
Sensitive		258	41.8%
Single resistant		209	33.9%
Multi resistant*		150	24.3%

* Resistance to one or more agent tested, quinolones were considered to be one group.

Salmonella isolates

The antimicrobial susceptibility of 94 *Salmonella* isolates, originating from 40 different broiler flocks, was determined. The numbers and percentages of resistant *Salmonella* isolates per antimicrobial agent are listed in Table 7.3. All isolates were resistant to erythromycin (control). A total of eight isolates, originating from three different broiler flocks, were resistant to nalidixic acid and flumequine (in combination with tetracycline), but not to ciprofloxacin or enrofloxacin. Eleven out of the 94 isolates (11.7%) were resistant to more than one agent tested.

Where possible, five *Campylobacter* and *Salmonella* isolates per flock were tested. Differences in susceptibility patterns in *Campylobacter* isolates from the same flock

Table 7.2. Antimicrobial susceptibility of 177 hippurate-positive and 91 hippurate-negative Campylobacter *isolates.*

Antimicrobial agent	Resistant isolates hippurate-positive number	%	Resistant isolates hippurate-negative number	%	*P*
Nalidixic acid only	2	1.0	2	2.0	NS*
All Quinolones	70	39.5	17	18.7	< 0.001
Quinolones**	5	2.8	10	11.0	< 0.01
Ampicillin	72	40.7	9	9.9	< 0.001
Tetracycline	21	11.9	17	18.7	NS
Erythromycin	3	1.7	11	12.1	< 0.001
None	61	34.5	49	53.8	< 0.005
Single	69	39.0	22	24.2	< 0.05
Multiple	47	26.5	20	22.0	NS

* NS Non-significant

** Resistant to nalidixic acid, flumequine, and ciprofloxacin. The diameter of the inhibition zone for enrofloxacin was 18-25 mm ('intermediate').

were found in 52% of the flocks. No particular similarity was found between the resistance patterns of *Campylobacter* and *Salmonella* isolates originating from the same broiler flock (38 cases).

Discussion

In this study the *in vitro* susceptibility of 617 *Campylobacter* isolates and 94 *Salmonella* isolates, all originating from poultry broiler flocks and isolated during

Table 7.3. Antimicrobial susceptibility of 94 Salmonella *isolates.*

Antimicrobial agent	Disc content	Resistant *Salmonella* isolates	
		number	percentage
Nalidixic acid	30 µg	8	8.5%
Flumequine	30 µg	8	8.5%
Ciprofloxacin	5 µg	0	0.0%
Enrofloxacin	5 µg	0	0.0%
Ampicillin	25 µg	5	5.3%
Tetracycline	30 µg	16	17.0%
Cephalothin	30 µg	1	1.1%
Erythromycin (control)	15 µg	94	100.0%
Sensitive		75	79.8%
Single resistant		8	8.5%
Multi resistant*		11	11.7%

* Resistance to one or more agent tested

the period March 1992 - March 1993, was examined. Special attention was given to the (fluoro)quinolones nalidixic acid, flumequine, ciprofloxacin, and enrofloxacin. The latter two agents recently became available for use in human and veterinary medicine in The Netherlands, respectively.

Campylobacter isolates showed almost complete cross-resistance to the quinolones tested. Cross-resistance between several fluoroquinolones and nalidixic acid was reported before (Endtz et al., 1991; Taylor et al., 1985). In 1989, Endtz et al. (1991) found 14% of 129 *Campylobacter* strains isolated from poultry products to be ciprofloxacin resistant. In the present study, 29.3% of 617 *Campylobacter* isolates were cross-resistant to the quinolones tested. These isolates originated from 38% of 150 broiler flocks, indicating an even more wide spread existence of quinolone resistant campylobacters. Quinolone resistance in *Campylobacter* isolates did not coincide with higher frequencies of resistance to ampicillin, tetracycline, or

erythromycin.

Of the 268 *Campylobacter* isolates, 66% were hippurate-positive (*C. jejuni*). The remaining 91 isolates were hippurate-negative, and most likely to be *C. coli* or perhaps *C. lari*, because of the isolation on CCD-agar (Bolton et al., 1984). A significantly ($P < 0.001$) higher percentage of quinolone resistant strains was found in the *C. jejuni* group (39.5%) than in the non-jejuni group (18.7%). Isolates from the latter group, however, were more often "intermediate resistant" to enrofloxacin ($P < 0.01$). Endtz et al. (1991) isolated comparable percentages of *C. jejuni* (72%) and *C. coli* (28%) from poultry products in 1989. But in contrast to our findings, resistance to the quinolones was observed more often in *C. coli* (14%) than in *C. jejuni* (6%).

Eight out of 94 (8.5%) *Salmonella* isolates in our study were found to be resistant to nalidixic acid and flumequine (in combination with tetracycline), but not to the fluoroquinolones ciprofloxacin or enrofloxacin. Up till now, poultry isolates of *Salmonella* generally show low (0-5%) resistance to nalidixic acid or fluoroquinolones (Salvat et al., 1992; Wray et al., 1992).

Quinolone (cross-)resistance in both *Salmonella* and *Campylobacter* isolates can have serious implications for the treatment of human diarrhoeal illness and causes problems in the identification of *Campylobacter* species. Additional tests, such as anaerobic growth in the presence of 1 g/l trimethylamine-N-oxide hydrochloride (TMAO), will have to be used to differentiate nalidixic acid resistant *C. coli* from *C. lari* (Endtz et al., 1991).

Though fluoroquinolones are becoming more popular, erythromycin still is the drug of choice when treatment of human campylobacteriosis is necessary (Hirschl et al., 1990; Rautelin et al., 1991). Erythromycin resistance in *Campylobacter* isolates was found to be 4.2%. This is in concordance with findings in other European countries (Hirschl et al., 1990; Rautelin et al., 1991; Reina et al., 1992). Erythromycin resistance is reported to be more frequent in *C. coli* than in *C. jejuni* (Rautelin et al., 1991; Taylor and Courvalin, 1988). In this study, 1.7% of 177 *C. jejuni* isolates and 12.1% of 91 *C. coli* isolates were erythromycin resistant ($P < 0.001$).

Where possible, five *Campylobacter* and *Salmonella* isolates per flocks were tested. Differences in patterns of susceptibility of *Campylobacter* isolates from the same flock were found in 52% of the flocks. This could indicate flock contamination with different *Campylobacter* types at the same time, as was also found with respect

to *Campylobacter* serotypes (Jacobs-Reitsma, 1992). This phenomenon was less pronounced for *Salmonella* isolates, but lower numbers of salmonellas were isolated and isolates showed less resistance.

Like enrofloxacin, ampicillin and (oxy)tetracycline are frequently used in Dutch broiler production. *Campylobacter* isolates showed considerable resistance to ampicillin and to a lesser extent towards tetracycline. In *Salmonella* isolates tetracycline resistance was more frequent than ampicillin resistance. No clear similarity with respect to their patterns of susceptibility was found between *Campylobacter* and *Salmonella* isolates originating from the same broiler flocks (38 cases). This might indicate that different mechanisms are involved in the development of resistance in *Campylobacter* and *Salmonella* bacteria. Detailed data on the medication of broiler flocks is necessary to provide more insight into the relation between the veterinary use of therapeutic agents and antimicrobial resistance in *Campylobacter* and *Salmonella* isolates.

CHAPTER 8

The Induction of Quinolone Resistance in *Campylobacter* Bacteria in Broilers by Quinolone Treatment

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ABSTRACT Induction of quinolone resistance in campylobacters by a quinolone treatment of *Campylobacter* colonized broilers was studied. Six groups of 15 broiler chicks each were administered a quinolone sensitive *C. jejuni* strain at 19 days of age. Starting at 26 days of age, two dosages (15 or 50 ppm) of flumequine or enrofloxacin were given via the drinking water for four days. One group was treated with enrofloxacin during the first four days of life. Quinolone treatment did not eradicate *Campylobacter* colonization in the broilers. On days 29, 33 and 43 (at slaughter) of life, chicks in both enrofloxacin treated groups harboured nalidixic acid, flumequine, and enrofloxacin resistant campylobacters. *Campylobacter* isolates from all other groups remained sensitive to these quinolones. Two *Campylobacter*-free control groups were not colonized by campylobacters during the whole experiment.

Introduction

Nowadays *Campylobacter* spp. are recognized as an important cause of human diarrhoea. Campylobacteriosis is mainly a food-borne infection and products of animal origin, in The Netherlands especially poultry meat, play a major role in transmission (Health Council of The Netherlands, 1988; Griffiths and Park, 1990). Most human *Campylobacter* infections are self-limiting and do not need antimicrobial therapy. However, in cases with severe symptoms or relapse medication is required. The drug of choice usually is erythromycin, although recently also fluoroquinolones, like ciprofloxacin or norfloxacin, are being used (Blaser et al., 1983; Hirschl et al., 1990).

Fluoroquinolones are chemically related to nalidixic acid, a "first generation" quinolone (Figure 8.1). Flumequine, a "second generation quinolone", has been in use in veterinary medicine since the early 1980s. In The Netherlands, enrofloxacin and ciprofloxacin (both "third generation" quinolones) were introduced into veterinary and human medicine in 1987 and 1988, respectively. Enrofloxacin is frequently used in broiler rearing in the first week of life to reduce vaccination problems or in the third or fourth week of age to combat respiratory problems due to *Escherichia coli*.

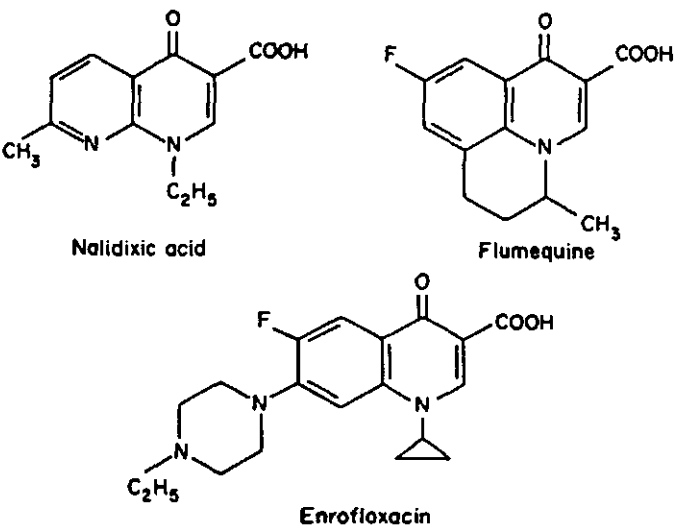

Figure 8.1. Chemical structure of the quinolones nalidixic acid, flumequine, and enrofloxacin.

A rise in ciprofloxacin resistant *Campylobacter* isolates from 0% in 1982 to 11% (human isolates) and 14% (poultry product isolates) was reported in 1989 (Endtz et al., 1991). In 1992 and 1993, Jacobs-Reitsma et al. (1993) found 181 out of 617 (29%) *Campylobacter* isolates to be cross-resistant to the quinolones nalidixic acid, flumequine, enrofloxacin and ciprofloxacin. In other European countries an increase in the number of quinolone-resistant *Campylobacter* isolates has also been reported (Rautelin et al., 1991; Reina et al., 1992).

Endtz et al. (1991) suggested that the rise in quinolone-resistant *Campylobacter* isolates in both humans and poultry products was related to the extensive use of quinolones in veterinary practice. However, quinolone-resistant campylobacters were also reported to appear in high frequency during human therapy (Wretlind et al., 1992; Segreti et al., 1992).

In this report, the induction of quinolone resistance in campylobacters by the quinolone treatment of *Campylobacter* colonized broilers was studied. Special attention was given to the possible cross-resistance between first, second, and third generation quinolones.

Materials and Methods

Experimental conditions

Day-old broiler chicks (Cobb) were obtained from a commercial hatchery. The parent flock of these chicks was screened for the presence of *Campylobacter* during the collection of hatching eggs. Eight groups of 15 chicks each were housed in floor-pens on litter. All groups were kept in separate rooms, except for groups 7 and 8 (*Campylobacter*-free controls) which were housed in the same room. Each room was provided with separate clothing and boots and a disinfection waterbath for the handlers. Tap water and standard pelleted broiler feed containing narasin as coccidiostat were provided *ad libitum*. The density of birds in the pens was comparable to commercial practice: 21 chicks/m^2 at the start and on day 36 this number was reduced to 16 chicks/m^2. The broiler chickens were spray-vaccinated at day of hatch against infectious bronchitis and Newcastle disease (NCD). On day 23 the NCD vaccination was repeated. Gumboro vaccination was given via the drinking water on day 17.

Quinolone treatment

The various treatments of the different chicken groups are given in Table 8.1. Quinolone treatment of the chickens lasted four days. Treatment of group 6 started at day 1 and treatment of groups 2-5 and 7 started at day 26. Commercially available stock solutions of 5% (w/v) Flumesol (active compound: flumequine) and 10% (w/v) Baytril® (active compound: enrofloxacin) were used (A.U.V., Cuijk, The Netherlands). Both agents were mixed in a concentration of 50 or 15 ppm in the drinking water. A concentration of 50 ppm in the drinking water equals the usual dosage of 10 mg/kg body weight per day. A fresh solution of the drinking water was prepared every treatment day.

Campylobacter challenge

Campylobacter challenge took place on day 19. All birds in groups 1-6 were individually challenged with 0.1 ml of saline solution containing $6 \cdot 10^7$ cfu/ml *Campylobacter* bacteria. The challenge strain *C. jejuni* COVP-356 was a broiler isolate with Penner serotype O2, and susceptible to the quinolones nalidixic acid, flumequine, and enrofloxacin. All birds in group 7 and 8 (*Campylobacter*-free control groups) were individually challenged with 0.1 ml of sterile saline solution.

Campylobacter sampling

On arrival the paperpads from the chicken transport crates of the day-old chicks were examined for the presence of *Campylobacter*. All groups were tested for the presence of *Campylobacter* on day 12, 19, 26, and 33 by individual cloacal swabbing. All chickens were slaughtered on day 43, and from each bird caecal material was aseptically sampled with a sterile swab. Groups 1-6 were additionally tested for the presence of *Campylobacter* on day 22 (three days after challenge) and on day 29 (last day of quinolone treatment). Swabs were streaked on *Campylobacter* Blood-Free Selective Medium (CCD-agar, Oxoid CM 739), with cefoperazone and amphotericin-B (Oxoid SR 155E) as selective agents. CCD-agar plates were incubated under micro-aerobic conditions (CampyPak Plus, BBL 71045). After two days of incubation at 37 °C, suspect colonies were

microscopically examined for typical corkscrew shaped organisms with a rapid, darting motility. Hippurate hydrolysis was tested using the method described by Hwang and Ederer (1975). Serotyping of isolates was performed according to Penner and Hennessy (1980).

Antimicrobial susceptibility testing

Material from the selective CCD-agar plates was subcultured on Blood agar (No. 2, Oxoid, CM 271) plates supplemented with 5% (v/v) sheep blood. Single colonies from the subculture were used in the antimicrobial susceptibility tests. Antimicrobial susceptibility was tested by the disc diffusion method (Bauer et al., 1966), using Mueller-Hinton agar plates (Oxoid, CM 337) supplemented with 5% (v/v) sheep blood. The following discs were used: cephalothin, 30 μg (Oxoid KF30); nalidixic acid, 30 μg (Oxoid NA30); flumequine, 30 μg (Oxoid UB30); and enrofloxacin, 5 μg (Bayer ENR5). Mueller-Hinton blood plates were incubated micro-aerobically at 37 °C. After two days, the diameter of the inhibition zones was measured with callipers. Isolates with an inhibition zone diameter ≤ 15 mm (including the disc diameter of 6 mm) were regarded as resistant. For enrofloxacin this criterion was ≤ 17 mm.

Results and Discussion

The parent flock of the broiler chicks in the experiment was found to be *Campylobacter* positive (*Campylobacter* isolated from 20/20 fresh caecal material samples). Serotyping of 10 isolates revealed eight isolates of Penner type O1,44 (*C. jejuni*) and two isolates of type O56 (*C. coli*) isolates. The challenge strain therefore was chosen to be of a different serotype (O2, *C. jejuni*). No *Campylobacter* bacteria were, however, isolated from the paperpads of the transport crates. Results of the *Campylobacter* sampling are presented in Table 8.1. All birds remained *Campylobacter*-free, unless deliberately challenged. Chicken groups 1-6 were readily colonized by *Campylobacter* strain COVP-356, all birds being *Campylobacter* positive three days after challenge. *Campylobacter* isolates were hippurate positive and serotype O2 throughout the whole experiment.

Table 8.1. Results of Campylobacter *swab samples and* Campylobacter *susceptibility testing per group and per sampling day.*

Group	Quinolone treatment (conc. ppm)	Treatment on days	day 19	day 22	day 26	day 29	day 33	day 43
Groups 1 to 6 were challenged with *C. jejuni* on day 19								
1	None		0/15*	15/15	6/6	12/14	14/14	12/12
				RSSS (3)†	RSSS (3)	RSSS (3)	RSSS (3)	RSSS (11)
2	Flumequine (15)	26-29	0/14	14/14	6/6	14/14	14/14	12/12
				RSSS (3)	RSSS (3)	RSSS (8)	RSSS (8)	RSSS (12)
3	Flumequine (50)	26-29	0/15	15/15	6/6	12/15	13/14	12/12
				RSSS (3)	RSSS (3)	RSSS (7)	RSSS (8)	RSSS (12)
4	Enrofloxacin (15)	26-29	0/15	15/15	6/6	14/15	15/15	12/12
				RSSS (3)	RSSS (3)	RRRR (8)	RRRR (8)	RRRR (12)
5	Enrofloxacin (50)	26-29	0/15	15/15	6/6	12/15	10/13	11/11
				RSSS (3)	RSSS (3)	RRRR (8)	RRRR (8)	RRRR (10)
6	Enrofloxacin (50)	1-4	0/15	15/15	14/14	14/14	13/14	12/12
				RSSS (13)	RSSS (13)	RSSS (3)	RSSS (3)	RSSS (12)
Control groups 7 and 8 were dosed with a sterile saline solution on day 19								
7	Enrofloxacin (50)	26-29	0/15	ND	0/15	ND	0/15	0/12
8	None		0/15	ND	0/15	ND	0/15	0/11

* Number of *Campylobacter*-colonized chickens/number of chickens tested.

† Number of *Campylobacter* isolates tested on susceptibility

RSSS, cephalothin^R, nalidixic acid^S, flumequine^S, enrofloxacin^S; RRRR, cephalothin^R, nalidixic acid^R, flumequine^R, enrofloxacin^R; ND, Not determined.

Quinolone treatment in this trial was not able to eradicate *Campylobacter* from the birds. Results of the antimicrobial susceptibility testing of *Campylobacter* isolates are summarized in Table 8.1. Campylobacters in chicks in group 1, not receiving any quinolone treatment, remained sensitive to the quinolones nalidixic acid, flumequine, and enrofloxacin. Chicks in group 6 were treated with Baytril® during the first four days of their life. *Campylobacter* colonization was accomplished only at day 26. This is about the age at which broilers become *Campylobacter* colonized in practice (Jacobs-Reitsma, 1992a). The early treatment of broilers with Baytril® did not induce quinolone resistance of the *Campylobacter* strain. This is in concordance with a field observation, that a broiler flock harboured quinolone-sensitive *Campylobacter* isolates at slaughter, although treated with Baytril® during the first week of life (Jacobs-Reitsma, 1992b). *Campylobacter* isolates from groups 2 and 3 did not notably change in their susceptibility to nalidixic acid, flumequine or enrofloxacin after treatment with flumequine at the two dosages. *Campylobacter* isolates from groups 4 and 5, however, were all cross-resistant to these quinolones after treatment with enrofloxacin, regardless of the dosage (Table 8.1). Three isolates from group 1 and six isolates from both groups 4 and 5 (all isolated on day 29) were subcultured at least 10 times on non-selective blood agar plates. Subculturing did not change the susceptibility pattern (Table 8.1, day 29).

The enrofloxacin treatment of *Campylobacter* colonized broiler chicks in this study led to cross-resistance in the challenge strain to nalidixic acid, flumequine, and enrofloxacin. This observation might be an explanation for the increasing number of quinolone (cross-)resistant *Campylobacter* isolates in poultry as reported by Endtz et al. (1991) and Jacobs-Reitsma et al. (1993). Thus the use of third generation quinolones in both animal husbandry and human medicine should be seriously re-assessed.

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CHAPTER 9

General Discussion

Introduction

Campylobacter bacteria are recognized as one of the most important causes of human diarrhoeal disease. It is estimated that each year in The Netherlands a total of 300,000 people suffer from a *Campylobacter* infection. Poultry products account for the majority of *Campylobacter* infections in humans. Reduction of *Campylobacter* contamination of poultry products will reduce the risk of campylobacteriosis to consumers.

Reduction of the potential risk of contaminated poultry products probably has to be achieved by a combination of Good Hygienic Practices (GHP) for both producers of poultry meat products and consumers of these products. The latter is a matter of education and adequate consumer information on proper handling of foods at risk. The former may need a combination of steps throughout the whole chain of the poultry production process. Because of the low infectious dose of *Campylobacter* in humans, it would be more effective to reduce the total number of carcasses contaminated, rather than to reduce the number of campylobacters on each carcass. Therefore, total prevention of *Campylobacter* colonization in live broiler flocks at the farm level is the most elegant way to reduce the contamination level of poultry products. At second hand, processing improvements at the slaughterhouse level and treatment of end-products might be considered to contribute in solving the problem. The aim of this thesis was to obtain a better understanding of the *Campylobacter* epidemiology in poultry in order to be able to formulate recommendations for reducing *Campylobacter* colonization in live broiler flocks. Several aspects as shown in Figure 1.1 (Chapter 1) were studied and results are discussed as illustrated in Figure 9.1.

Serotyping as a tool in epidemiological studies

Typing methods for detailed strain characterization are essential in studies on *Campylobacter* epidemiology in order to provide useful information for identifying sources and modes of transmission. One of the best known and most widely used typing methods for *Campylobacter* is the serotyping method based on heat-stable antigens as described by Penner and Hennessy (1980). Recent developments in

molecular typing methods for *Campylobacter* look promising. However, in spite of the fact that these new methods are genotypic methods, they lack the advantage of the serotyping system, as being internationally recognized and providing data that can be compared among laboratories.

The Penner serotyping system with the modifications of using absorbed and pooled sera as described in Chapter 2, was easy to work with in routine sampling. However, the availability of sera is crucial; when production and efficiency testing of new batches of absorbed sera has to be included, the system requires much more work and time.

Over 80% of the *Campylobacter* isolates in our studies were typable with the modified Penner serotyping system (Chapter 2). Typability of strains was clearly affected by storage of the strains before actual typing. Typability of frozen stored isolates could be improved by additional subculturings on blood agar plates, but this leads to an undesirable extra effort in work and time. As extracted antigens appeared to be stable for at least six months at 4 °C, it is advisable to store extracted antigens from freshly isolated *Campylobacter* strains instead of reculturing frozen stored strains, when actual typing can not be performed directly after primary isolation.

Untypability of isolates may partly be explained by the detection of *Campylobacter* serotypes currently not represented in the serotyping system. Indications for this were observed in at least two different studies (Chapters 2 and 5). These untypable strains might be useful for extending the typing system.

Experiments on repeated serotyping of several *Campylobacter* strains did not suggest any serotype instability within the strains, except maybe for the "instability" of being untypable after cold-storage, but typable after several subculturings (Chapter 2). Also after induction of quinolone resistance as described in Chapter 8, the serotype of the *Campylobacter* isolates remained identical to the initial serotype O2.

Humans usually seem to be infected with a single *Campylobacter* serotype, although also mixed infections are reported (Patton and Wachmuth, 1992; Albert et al., 1992). However, healthy carriers like poultry often harbour quite a number of different serotypes at a time. More than five different serotypes could be isolated from one broiler, breeder or layer flock (Chapters 3, 4, and 5). This diversity was also observed in the antimicrobial susceptibility patterns of *Campylobacter* isolates

within broiler flocks (Chapter 7). This large variety in *Campylobacter* strains most likely reflects the continuous flow of new campylobacters entering a poultry flock. Mixed contamination of poultry flocks may be enforced by the fact that potential contamination sources also may harbour a variety of *Campylobacter* strains, as was observed in pigs, cattle and sheep (Chapter 5). Serotype dominance of newly introduced strains may lead to a change in serotype patterns during a broiler production cycle (Chapter 5). Laboratory experiments with broilers and challenge of two different *Campylobacter* serotype strains showed a complete dominance of one type over the other within one week (Jacobs-Reitsma, 1992c).
The observations on the large variety of *Campylobacter* serotypes in animal reservoirs and the possibility of serotype dominance indicate the complexity of *Campylobacter* epidemiology in poultry. The importance of the number of isolates that is tested might easily be underestimated in investigations on *Campylobacter* epidemiology, but this certainly needs special attention in the design of this type of investigations.

Campylobacter epidemiology in poultry

Prevalence and seasonal variation of *Campylobacter* colonization in broiler flocks

Results in Chapter 6 clearly indicate a high caecal carriage rate of *Campylobacter* (82%) and a lower carriage rate of *Salmonella* (27%) in Dutch broiler flocks at slaughter during the period March 1992 to March 1993. The high incidence of *Campylobacter* in caeca at slaughter implicate a high risk for contamination of broiler carcasses during processing (Mead and Hinton, 1989; Shane, 1992).
Campylobacter carriage in the broiler flocks showed seasonal variation, with the highest contamination rate (100%) during the period from June to September and the lowest (50%) in March (Chapter 6). Contamination of broiler flocks with *Campylobacter* occurred earlier in the production cycle during summer than during winter and the three broiler farms that remained *Campylobacter* negative for two production cycles in the preliminary study were screened during late autumn or the winter period (Chapter 5).

The consistency of this seasonal variation in *Campylobacter* prevalence in broiler flocks has to be confirmed by repeated screening over a period of at least another year.

Jones et al. (1990) suggested that the summertime peak yearly found in human campylobacteriosis was related to a peak in *Campylobacter* colonization in animals. The importance of a seasonal variation in *Campylobacter* prevalence in broilers for *Campylobacter* epidemiology in poultry has to be determined more closely and might include a possible role of migratory birds or a seasonal presence or activity of certain insects. Certainly, the seasonal variation in *Campylobacter* prevalence in broiler flocks should be taken into account when broiler flocks are studied to evaluate implementation of hygienic control measures at the farm level.

Overall, prevalence of *Campylobacter* on the two broiler farms in Chapter 5 were 56 and 91%, respectively and *Campylobacter* generally was isolated for the first time in a flock between three and four weeks of age. No campylobacters were ever detected before the birds were two weeks of age. These findings are in agreement with other longitudinal studies, where the earliest detection time of *Campylobacter* in a broiler flock is not reported in the first two weeks either (Altmeyer et al., 1985; Engvall et al., 1986; Pokamunski et al., 1986). The reason for this phenomenon remains unclear. Suggestions have been made as to either a level of *Campylobacter* below the detection limit or protection via maternal immunity. However, challenge experiments with day-old chicks do not support these theories. Both orally dosed or challenge via seeder birds did easily lead to *Campylobacter* colonization of day-old chicks and *Campylobacter* could easily be isolated in faecal samples in as early as two days after challenge, even if challenge doses were low (Shanker et al., 1990; Bolder and Mulder, 1991).

Once *Campylobacter* was isolated from a flock, all broilers in the particular broiler house appeared to be colonized within one week and isolation rates in the flocks remained at 100% up to slaughter (Chapter 5). Similar findings of rapid horizontal spread and high isolation rates within broiler flocks are reported in several other studies (Engvall et al., 1986; Pokamunski et al., 1986). Coprophagy may explain the rapid transmission of *Campylobacter* between the broilers within a house, but also water, feed, litter and even air may help to spread the organism, once it is present in a flock (Evans, 1992).

Vertical transmission

The studies on breeder flocks (Chapter 3) and on laying hens (Chapter 4) revealed that healthy carriage of *Campylobacter* in these parts of the poultry production chain is also quite common. *Campylobacter* colonized breeder flocks implicate a potential role for a vertical transmission via the egg and hatchery to progeny. However, in addition to the findings in other studies (Shanker et al., 1986; Annan-Prah and Janc, 1988; Van de Giessen et al., 1992), results do not suggest an important role for vertical transmission.

Observations in the longitudinal study on two broiler production farms (Chapter 5) did not support a vertical transmission route, either. No *Campylobacter* was isolated from any of the hatchery samples examined and presence of *Campylobacter* in the broiler flocks could not be demonstrated before a minimum of two weeks of life. Broilers from identical parent flocks were found to be colonized in one production cycle and *Campylobacter*-free in another cycle. Even if broilers were colonized, the serotype pattern was clearly more associated with the production cycle itself (and the other houses on the farm), than to the various origins of the broiler flocks during that cycle.

Certainly, breeder flocks and laying hens have to be recognized as another reservoir of *Campylobacter*. However, it might be more appropriate to consider this as a potential risk factor in horizontal transmission routes rather than as a risk factor for vertical transmission via the egg.

The *Campylobacter* isolation from four out of 100 ovaries from naturally contaminated laying hens was in contrast with the absence of the organism in a total of 219 egg yolks from freshly laid eggs originating from the same layer flocks (Chapter 4). The presence of *Campylobacter* in organs of the reproductive tract of these laying hens seemed to originate from an external (faecal) contamination, rather than from an internal invasion via the blood or lymph. Nevertheless, the impact of this type of *Campylobacter* contamination of laying hens has to be determined more closely.

Horizontal transmission from one production cycle to the next

Figure 1.1 shows a broiler house that is assumed to be *Campylobacter*-free at start. The possibility of *Campylobacter* transmission from one flock to the next via used

litter was reported by Montrose et al. (1985). However, the use of old litter is no common practice in The Netherlands. Broiler houses in The Netherlands are totally emptied after each production period and often also thoroughly cleaned and disinfected. Dutch law requires that houses remain empty for at least seven days between crops. The two farms in Chapter 5 were working according to this principle and *Campylobacter* isolation and serotyping results do not suggest that *Campylobacter* was transmitted from one cycle to the next via a persistent contamination of the broiler house. Consecutive production cycles generally showed a different serotype pattern and also *Campylobacter* negative flocks were observed, following *Campylobacter* positive ones.

Campylobacter contamination of manure, used litter, and waste water after cleaning was not established in our studies (Figure 9.1). However, these factors are likely to be contaminated with *Campylobacter* and may play a role in maintenance of the *Campylobacter* contamination cycle in the environment.

Results presented in both Chapter 5 and Chapter 6 indicated a high prevalence of *Campylobacter* in broiler flocks, and a high isolation percentage of the organism within each flock. So, broilers in transport crates leaving the production farm for slaughter in fact represent the main risk factor for contamination of poultry meat products.

Horizontal transmission from the environment

As described in Chapter 5, *Campylobacter* was not isolated from any of the fresh litter (straw) or feed samples. Both litter material and feed are generally considered to be unsuitable environments for *Campylobacter* to survive, because of the low water activity (Evans, 1992).

The fresh drinking water samples from the tap water reservoirs on the two farms were not found to be contaminated with *Campylobacter*. However, when broilers in the house were *Campylobacter*-colonized, *Campylobacter* could also be isolated from (bell-type) drinkers inside the houses.

Several authors found the use of untreated well water for feeding broilers to be an important risk factor in *Campylobacter* colonization of broiler flocks (Pearson et al., 1993; Kapperud et al., 1993). In our study, only six farms (10%) were using untreated well water instead of tap water. Although all six farms harboured

Campylobacter-positive broiler flocks, the number of farms was too low to reach statistical significance (Chapter 6).

Sampling of air was not included in our studies. Several authors report *Campylobacter* isolation from aerosols frequently present in slaughterhouses (Oosterom et al., 1983a; Mead and Hinton, 1989), but also from air samples on a broiler farm (Engvall et al., 1986).

Though survival conditions for *Campylobacter* in air do not seem to be favourable, air-borne *Campylobacter* colonization might explain the spread of *Campylobacter* throughout poultry houses with laying hens in battery cages (Chapter 4).

Campylobacter isolations from transport crates are reported (Hoop and Ehrsam, 1987), sometimes even after cleaning and disinfection. This finding is important in slaughterhouse hygiene programmes, and crates have to be regarded as a potential source of *Campylobacter* contamination at the farm level, especially in a system of partial depopulation at five weeks of age, whereas the remainder of the flock is slaughtered at about six weeks of age.

The relatively late contamination with *Campylobacter* in cycle 1 on Farm D and cycle 3 on Farm E (Chapter 5) might have been due to this quite common Dutch practice of partial depopulation. Efforts on strict hygienic management during the whole production period may be spoiled by introducing a late, but preventable, *Campylobacter* contamination just before slaughter.

Healthy carriage of *Campylobacter* in humans is hardly ever found, so farmers are not regarded as direct sources of transmission. Indirect transmission via boots or utensils, however, might easily occur. Especially when potential sources of *Campylobacter* on the farm are present. Farm animals like sheep, cattle, and especially pigs and laying hens, were found to be more or less permanent carriers of *Campylobacter* (Chapter 5). Serotypes isolated from these animals were in most, but certainly not all, cases different from serotypes found in the broilers at the same time. Nevertheless, these domestic animals have to be regarded as important sources of *Campylobacter* contamination for broilers. Kapperud et al. (1993) found pig production to be significantly associated with the presence of *Campylobacter* in broiler flocks. As noted in Chapter 6, 22% of the 59 broiler farmers were also keeping pigs on their farms. No statistically significant association was found between keeping pigs and the presence of *Campylobacter* in the broiler flocks. However, 34% of 268 *Campylobacter* isolates from the broiler flocks at slaughter

were *Campylobacter coli*. As *C. coli* is highly associated with pigs (Skirrow and Blaser, 1992), *Campylobacter coli* isolation from broiler flocks might indicate contamination from pigs.
Rosef and Kapperud (1983) isolated *Campylobacter* from more than 50% of a total of 146 flies captured in the surroundings of a poultry production farm. Jones et al. (1992), however, could not detect internal *Campylobacter* contamination in insects such as flies, beetles or cockroaches. It was reported in Chapter 5 that on several occasions *Campylobacter* was isolated from the internal contents of darkling beetles and lesser mealworms, although this was not before the organism was also isolated from the broilers. However, identical serotypes were isolated from both the insects and broilers within the broiler houses. This might indicate an infection route from insects to broilers, but the reverse infection route from broilers to insects is just as likely. More detailed studies are needed to determine the survival and colonization potentials of *Campylobacter* in these insects.

Quinolone resistance in *Campylobacter* isolates from poultry

Another aspect of *Campylobacter* epidemiology in poultry, which may have serious implications for the treatment of human diarrhoeal illness, is the development of resistance against quinolones in *Campylobacter* isolates.
Fluoroquinolones are chemically related to nalidixic acid, a 'first generation' quinolone. Flumequine, a 'second generation quinolone', has been in use in veterinary medicine since the early 1980's. In The Netherlands, enrofloxacin and ciprofloxacin (both 'third generation' quinolones) were introduced into veterinary and human medicine in 1987 and 1988, respectively. Enrofloxacin is frequently used in broiler rearing in the first week of life to reduce vaccination problems or in the third or fourth week to combat respiratory problems due to *Escherichia coli*.
Endtz et al. (1991) reported a rise in ciprofloxacin resistant *Campylobacter* isolates from 0% in 1982 to 11% (human isolates) and 14% (poultry product isolates) in 1989. In our study in 1992 and 1993 (Chapter 7), already 29% of 617 *Campylobacter* isolates were found to be cross-resistant to the quinolones nalidixic acid, flumequine, enrofloxacin and ciprofloxacin. Endtz et al. (1991) suggested that the rise in quinolone resistant *Campylobacter* isolates in both humans and poultry

products was related to the extensive use of quinolones in veterinary practice. Detailed data on the medication of broiler flocks are necessary to provide more insight into the relation between the veterinary use of therapeutic agents and antimicrobial resistance in *Campylobacter* isolates.

Nevertheless, enrofloxacin treatment of *Campylobacter* colonized broiler chicks almost instantly led to cross-resistance in the challenge strain to nalidixic acid, flumequine, and enrofloxacin (Chapter 8). Also during human therapy, quinolone resistant campylobacters were reported to appear in high frequency (Wretlind et al., 1992; Segreti et al., 1992).

Thus the use of third generation quinolones in both animal husbandry and human medicine should be seriously reassessed.

Conclusions and recommendations

Results from both Chapters 5 and 6 indicate that *Campylobacter*-free rearing of broiler flocks can be a reality. Therefore, this has to be regarded as an important tool in the prevention of human campylobacteriosis via poultry meat products.

Figure 9.1 shows the modification of the initial flow diagram of a broiler house with possible transmission routes for *Campylobacter* contamination, based on the results as presented in this thesis.

No evidence was found for vertical transmission from breeder flocks via the hatchery to progeny, nor for a horizontal transmission from one broiler flock to the next via a persistent contamination within the broiler house.

By far the major route for *Campylobacter* contamination of poultry is a horizontal transmission from the environment. It is anticipated that carry-over from environmental contamination sources into the broiler house is caused by the farmer via the boots or other equipment. Pigs and poultry flocks (broilers, as well as laying hens and breeders), and to a lesser extent, sheep and cattle were found to be potential sources of *Campylobacter* contamination.

Quinolone treatment should not be seen as an answer to the problem of eradication of *Campylobacter* contamination from poultry flocks.

Horizontal intervention procedures at the farm level have to be studied further and the effectiveness of strict hygienic practices during the whole production period,

such as was described as being successful in small scale experiments by Van de Giessen et al. (1992) and Humphrey et al. (1993), has to be evaluated on a larger scale. Special attention has to be given to the influence of different housing systems and effects of transport on *Campylobacter* colonization in broilers.

Future research should also include studies on the phenomenon that broilers do not become *Campylobacter*-positive before a minimum of two weeks of age, and investigations on the differences in susceptibility towards *Campylobacter* colonization in different broiler breeds. This knowledge may be useful in further intervention procedures at the farm level.

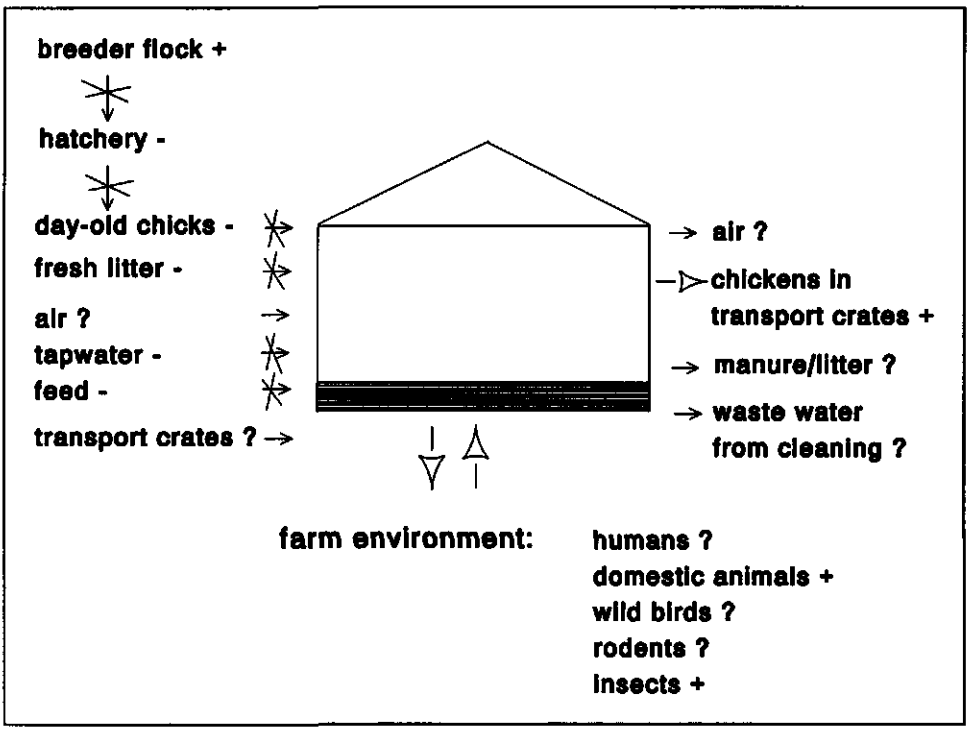

Figure 9.1 *Flow diagram of possible transmission routes for* Campylobacter *in a broiler house, based on the results as described in this thesis.*
+ : Campylobacter *isolations;* - : *no* Campylobacter *isolations;*
? : not determined; : *no* Campylobacter *transmission route;*
—▷ : *main* Campylobacter *transmission route.*

Summary

Epidemiology of *Campylobacter* in poultry

Campylobacter bacteria are recognized as one of the most important causes of human diarrhoeal disease. It is estimated that each year in The Netherlands a total of 300,000 people suffer from a *Campylobacter* infection. Apart from the human suffering, the economic impact due to lost productivity and health care of this number of *Campylobacter* infections is considerable. Poultry products account for the majority of *Campylobacter* infections in humans. Reduction of *Campylobacter* contamination of poultry products will reduce the risk of campylobacteriosis to consumers. Prevention of *Campylobacter* colonization in live broiler flocks at the farm level is the most elegant and efficient way to reduce the contamination level of poultry products. The aim of the work presented in this thesis was to obtain a better understanding of *Campylobacter* epidemiology in poultry, in order to be able to formulate recommendations to reduce *Campylobacter* colonization in live broiler flocks.

The Penner serotyping system was used as the epidemiological marker in most of the studies described in this thesis. However, the original method was modified to include absorption of antisera and the use of a pooled typing system and these modifications are described and discussed in Chapter 2. Over 80% of the *Campylobacter* isolates in our studies were typable with this modified Penner serotyping system. Non-typability of isolates may partly be explained by the detection of *Campylobacter* serotypes not yet represented in the serotyping system. Experiments on repeated serotyping of several *Campylobacter* strains did not suggest any serotype instability within the strains. Typability of strains was clearly affected by storage of the strains before actual typing. It is therefore advisable to store extracted antigens from freshly isolated *Campylobacter* strains instead of reculturing frozen stored strains, when actual typing can not be performed directly after primary isolation.

A screening study on *Campylobacter* colonization in breeder flocks is described in Chapter 3. Nine Dutch breeder farms with a total of 43 separately housed flocks were screened for the presence of *Campylobacter* spp. and *Salmonella* spp. *Campylobacter* was isolated from 29 of the flocks (67%) and *Salmonella* from 12

flocks (28%). Two of the nine farms were both *Campylobacter*- and *Salmonella*-free at the time of sampling. Two other farms were *Salmonella*-free, but were *Campylobacter*-positive. A total of 330 *Campylobacter* isolates were serotyped and 19 different *Campylobacter* serotypes were observed. A similar pattern of serotypes was generally present in all flocks on a farm.

Campylobacter colonization of laying hens is described in Chapter 4. Two groups of laying hens, housed in battery cages or in aviaries, were screened for presence of *Campylobacter* during their productive life. Layers in both housing systems became colonized with *Campylobacter*, though contamination in battery-housed flocks was delayed. Isolation rates within positive flocks, however, were close to 100% and layers became permanent excreters of *Campylobacter* in their caecal droppings up to the time of slaughter. Penner serotyping of *Campylobacter* isolates indicated horizontal transmission from the environment. From three out of 179 eggs from *Campylobacter* excreting flocks, *Campylobacter* was isolated from faecally contaminated shell surfaces. However, *Campylobacter* was not isolated from the yolks of these eggs nor from a further 40 yolks examined. At slaughter, *Campylobacter* was isolated from the caeca of 93 out of the 100 birds tested. *Campylobacter* was also isolated from samples of ovaries (4x), magnum (7x), isthmus (11x), and uterus (14x), with a total of 21 birds having *Campylobacter* contamination in the reproductive tract. This contamination most likely originated from an external (faecal) contamination.

Chapter 5 describes a longitudinal survey on two Dutch poultry farms. Broiler flocks were screened weekly for the presence of *Campylobacter* in fresh caecal droppings during eight consecutive production cycles. Hatchery and fresh litter samples were taken at the start of each new cycle. Water, feed, insects, and faeces of domestic animals present on the farms were also included in the sampling. Generally, broiler flocks became colonized with *Campylobacter* at about three to four weeks of age and remained colonized up to the time of slaughter. *Campylobacter* isolation rates within flocks were close to 100%. Similar serotypes were found within the various broiler houses on one farm during one production cycle. New flocks generally showed a new pattern of serotypes. Most, but not all, serotypes isolated from laying hens, pigs, sheep, and cattle were different from those isolated from the broilers at the same time. *Campylobacter* serotypes from darkling beetles found inside the broiler houses were identical to the ones isolated from the broilers.

Campylobacter was not isolated from any of the hatchery, fresh litter, water or feed samples.

Chapter 6 describes a one-year study on the seasonal prevalence of *Campylobacter* and *Salmonella* in caecal samples taken from broilers at slaughter. A total of 187 Dutch broiler flocks, at three different slaughterhouses, were screened during the period from March 1992 to March 1993. At least 10 flocks were sampled every four weeks and 25 caecal samples were taken from each flock. *Campylobacter* spp. were isolated from 153 of the flocks (82%). *Campylobacter* carriage of flocks showed seasonal variation, with the highest contamination rate (100%) during the period June to September and the lowest (50%) in March. *Salmonella* carriage of the flocks did not show a distinct seasonal variation. *Salmonella* spp. were isolated from 49 out of 181 broiler flocks (27%). A positive correlation was found between *Campylobacter* and *Salmonella* colonization within flocks.

A screening for antibiotic resistance, especially to quinolones, was included in the one-year study on the prevalence of *Campylobacter* and *Salmonella* at slaughter and results are presented in Chapter 7. 617 *Campylobacter* isolates from 150 broiler flocks were tested for their susceptibility to cephalothin (control), ampicillin, tetracycline, erythromycin, and the quinolones nalidixic acid, flumequine, enrofloxacin, and ciprofloxacin by a disc diffusion method. *Campylobacter* isolates showed almost complete cross-resistance between the quinolones tested. 181 *Campylobacter* isolates (29%), originating from 55 flocks (37%), were quinolone resistant. 94 *Salmonella* isolates from 40 flocks were also tested for their antimicrobial susceptibility. Eight isolates (8.5%), from three broiler flocks (7.5%), showed resistance to nalidixic acid and flumequine (and tetracycline), but not to ciprofloxacin or enrofloxacin.

Results of a study on induction of quinolone resistance in campylobacters by a quinolone treatment of *Campylobacter*-colonized broilers are described in Chapter 8. Six groups of 15 broiler chicks each were administered a quinolone sensitive *C. jejuni* strain at 19 days of age. Starting at 26 days of age, two dosages (15 or 50 ppm) of flumequine or enrofloxacin were given via the drinking water for four days. One group was treated with enrofloxacin during the first four days of life. Quinolone treatment did not eradicate *Campylobacter* colonization in the broilers. On days 29, 33 and 43 (at slaughter), chicks in both enrofloxacin treated groups harboured campylobacters resistant to nalidixic acid, flumequine, and enrofloxacin.

Campylobacter isolates from all other groups remained sensitive to these quinolones.

A general discussion of the work presented in this thesis is given in Chapter 9. Figure 9.1 shows the modification of the initial flow diagram of a broiler house with possible transmission routes for *Campylobacter* contamination, based on the results as presented in this thesis.

No evidence was found for vertical transmission from breeder flocks via the hatchery to progeny, nor for a horizontal transmission from one broiler flock to the next via a persistent house-contamination.

By far the major route for *Campylobacter* contamination of poultry is a horizontal transmission from the environment. It is anticipated that carry-over from environmental contamination sources into the broiler house is caused by the farmer via the boots or other equipment. Pigs and poultry flocks (broilers, as well as laying hens and breeders), and to a lesser extent, sheep and cattle were found to be potential sources of *Campylobacter* contamination.

Quinolone treatment should not be seen as an answer to the problem of eradication of *Campylobacter* contamination from poultry flocks.

Horizontal intervention procedures at the farm level have to be studied further and the effectiveness of strict hygienic practices during the whole production period has to be evaluated on a larger scale.

Samenvatting

Epidemiologie van *Campylobacter* bij pluimvee

Campylobacter bacteriën zijn een van de belangrijkste veroorzakers van diarree bij de mens. Geschat wordt dat er jaarlijks in Nederland ongeveer 300.000 mensen een *Campylobacter* infectie doormaken. Nog afgezien van de persoonlijke ellende, is de economische schadepost ten gevolge van gemaakte ziektekosten en het verlies aan arbeidsproduktiviteit aanzienlijk. Pluimveevleesprodukten zijn verantwoordelijk voor het merendeel van de *Campylobacter* infecties bij de mens. Campylobacteriose bij de mens kan worden teruggedrongen door het terugdringen van de *Campylobacter* besmetting van pluimveevleesprodukten. De meest elegante en efficiënte manier om *Campylobacter* besmetting van pluimveevleesprodukten terug te dringen is het voorkómen van *Campylobacter* besmetting van levend pluimvee.

Het doel van het in dit proefschrift beschreven onderzoek was het verkrijgen van een beter inzicht in de epidemiologie van *Campylobacter* bij pluimvee, om op basis hiervan maatregelen te kunnen nemen om de *Campylobacter* besmetting bij levend pluimvee terug te dringen.

In de meeste studies in dit proefschrift werd het Penner serotyperingssysteem als epidemiologische marker gebruikt. De absorptie van antisera en het gebruik van een gepooled systeem werden geïntroduceerd en deze aanpassingen in de oorspronkelijke methode worden beschreven in Hoofdstuk 2. Met dit aangepaste Penner serotyperingssysteem was ruim 80% van de *Campylobacter* isolaten uit het onderzoek typeerbaar. Ontypeerbaarheid van isolaten zou deels kunnen worden verklaard door de aanwezigheid van *Campylobacter* serotypen die tot nu toe nog niet in het serotyperingssysteem waren opgenomen. Herhaaldelijk serotyperen van een aantal *Campylobacter* stammen leverde geen aanwijzingen op voor een mogelijke instabiliteit van het serotype binnen deze stammen. Het bij -80°C bewaren van *Campylobacter* isolaten had een duidelijke invloed op de typeerbaarheid. Wanneer serotyperen niet direct na isolatie kan worden uitgevoerd is het daarom aan te raden om de geëxtraheerde antigenen van verse *Campylobacter* isolaten te bewaren, in plaats van het opkweken van diepgevroren isolaten.

Hoofdstuk 3 beschrijft een onderzoek naar het vóórkomen van *Campylobacter* bij vermeerderingskoppels. Negen bedrijven met ouderdieren, met in totaal 43

gescheiden gehuisveste koppels, werden onderzocht op aanwezigheid van *Campylobacter* en *Salmonella*. *Campylobacter* werd geïsoleerd uit 29 van de 43 koppels (67%) en *Salmonella* werd geïsoleerd uit 12 koppels (28%). Twee van de negen bedrijven waren op het tijdstip van onderzoek vrij van zowel *Campylobacter* als *Salmonella*. Twee andere bedrijven waren *Salmonella*-vrij, maar *Campylobacter*-positief. In totaal werden 330 *Campylobacter* isolaten geserotypeerd en in dit onderzoek werden 19 verschillende serotypen aangetroffen. Per bedrijf werd in alle aanwezige koppels een overeenkomstig patroon van serotypen gevonden.

Hoofdstuk 4 beschrijft een onderzoek naar *Campylobacter* besmetting bij leghennen. Twee groepen leghennen, gehuisvest in batterijen of op de grond, werden gedurende hun (produktieve) leven onderzocht op aanwezigheid van *Campylobacter*. De leghennen in beide huisvestingssystemen raakten besmet met *Campylobacter*, maar de *Campylobacter* besmetting in de koppels die op batterijen gehuisvest waren, trad later op. Het isolatiepercentage binnen positieve koppels was vrijwel 100% en de leghennen bleven tot aan de slacht steeds *Campylobacter* uitscheiden via hun blinde darmmest. Serotypering van *Campylobacter* isolaten wees in de richting van een horizontale besmetting vanuit de omgeving. Van drie van de 179 eieren, afkomstig van *Campylobacter* uitscheidende legkoppels, werd *Campylobacter* geïsoleerd van met mest bevuilde eischalen, maar niet uit de eidooiers, of uit 40 aanvullend onderzochte eidooiers. *Campylobacter* werd geïsoleerd uit de blinde darmen van 93 van 100 nader onderzochte leghennen. *Campylobacter* werd ook aangetroffen in ovaria (4x), magnum (7x), istmus (11x) en uterus (14x), waarbij in totaal bij 21 dieren een *Campylobacter* besmetting in het legapparaat werd gevonden. Waarschijnlijk was deze besmetting afkomstig van een faecale besmetting van buitenaf.

Hoofdstuk 5 beschrijft een longitudinale studie op twee Nederlandse pluimvee-bedrijven. Vleeskuikenkoppels werden gedurende acht produktieronden wekelijks onderzocht op aanwezigheid van *Campylobacter* in verse blinde darmmest. Broederij afval en vers strooisel werd aan het begin van elke nieuwe ronde onderzocht. Ook water, voer, insekten en mest van landbouwhuisdieren die op de bedrijven aanwezig waren, werden bemonsterd. In het algemeen raakte een vleeskuikenkoppel op een leeftijd van drie tot vier weken besmet met *Campylobacter* en bleef besmet tot aan de slacht. Het isolatiepercentage binnen besmette koppels lag tegen de 100%. Tijdens een produktieperiode werden

overeenkomstige patronen van *Campylobacter* serotypen gevonden in de verschillende stallen op een bedrijf. Volgende koppels vertoonden in het algemeen weer een nieuw patroon van serotypen. Veel, maar zeker niet alle, serotypen die uit de leghennen, varkens, schapen en runderen werden geïsoleerd verschilden van de serotypen die tegelijkertijd in de vleeskuikens werden aangetroffen. *Campylobacter* serotypen die uit tempexkevers in de stallen werden geïsoleerd kwamen overeen met de serotypen die uit de vleeskuikens werden geïsoleerd. *Campylobacter* werd geen enkele keer aangetroffen in het broederij afval, vers strooisel, water of voer.
Hoofdstuk 6 beschrijft een onderzoek naar het seizoensgebonden vóórkomen van *Campylobacter* en *Salmonella* in blinde darmmonsters van vleeskuikens aan de slachtlijn. Van maart 1992 tot en met maart 1993 werden in totaal 187 vleeskuikenkoppels onderzocht, afkomstig van drie verschillende slachterijen. Elke vier weken werden minimaal 10 koppels bemonsterd en per koppel werden 25 blinde darmen onderzocht. Uit 153 van de 187 koppels werd *Campylobacter* geïsoleerd. De *Campylobacter* besmetting van vleeskuikenkoppels vertoonde een seizoensinvloed, met de hoogste besmettingsgraad (100%) gedurende de maanden juni tot en met september, en de laagste besmettingsgraad in maart (50%). *Salmonella* besmetting van de koppels vertoonde geen duidelijke seizoensinvloed. In 49 van de 181 vleeskuikenkoppels werd *Salmonella* aangetroffen. Er werd een positieve samenhang gevonden tussen het voorkomen van *Campylobacter* en *Salmonella* binnen koppels.
Hoofdstuk 7 beschrijft de resultaten van een onderzoek naar resistentie tegen antibiotica, met name tegen quinolonen. Met behulp van een schijfjes-diffusie methode werden 617 *Campylobacter* isolaten, afkomstig uit 150 vleeskuikenkoppels, onderzocht op gevoeligheid voor cephalotine (controle), ampicilline, tetracycline, erythromycine en de quinolonen nalidixinezuur, flumequine, enrofloxacin en ciprofloxacin. Bij *Campylobacter* isolaten werd een vrijwel volledige kruisresistentie gevonden tussen de onderzochte quinolonen. 181 *Campylobacter* isolaten (29%), afkomstig uit 55 vleeskuikenkoppels (37%), waren resistent tegen de quinolonen. Ook 94 *Salmonella* isolaten, afkomstig uit 40 koppels, werden onderzocht op antibioticum gevoeligheid. Acht isolaten (8,5%), uit drie koppels (7,5%), waren resistent tegen nalidixinezuur en flumequine (en tetracycline), maar niet tegen enrofloxacin of ciprofloxacin.
De resultaten van een onderzoek naar inductie van quinoloon resistentie in

campylobacters door een quinoloon behandeling van met *Campylobacter* besmette vleeskuikens worden beschreven in Hoofdstuk 8. Op de leeftijd van 19 dagen kregen zes groepen van elk 15 kuikens een quinoloon-gevoelige *C. jejuni* stam toegediend. Vanaf dag 26 werd gedurende vier dagen flumequine of enrofloxacin via het drinkwater toegediend, elk in een concentratie van 15 of 50 ppm. Eén groep kuikens werd gedurende de eerste vier levensdagen behandeld met enrofloxacin en de controlegroep kreeg geen quinoloon behandeling. De behandeling met quinolonen deed de *Campylobacter* besmetting van de kuikens niet verdwijnen. Uit beide met enrofloxacin behandelde groepen kuikens waarbij op dag 26 werd gestart met de behandeling, werden op dag 29, 33 en 43 (bij de slacht) campylobacters geïsoleerd die resistent waren tegen nalidixinezuur, flumequine en enrofloxacin. *Campylobacter* isolaten uit de overige vier groepen kuikens bleven gevoelig voor deze quinolonen.

Een algemene discussie van de resultaten van de verschillende onderzoeken wordt gegeven in Hoofdstuk 9. Figuur 9.1 geeft het flow-diagram van een vleeskuikenstal met mogelijke transmissieroutes voor *Campylobacter* besmetting weer, aangepast aan de resultaten beschreven in dit proefschrift.

Er is geen bewijs gevonden voor het bestaan van een verticale overdracht van *Campylobacter* door ouderdieren via de broederij naar hun nageslacht, noch voor een horizontale overdracht van het ene vleeskuikenkoppel naar het volgende via een blijvende besmetting van de kuikenstal.

De belangrijkste *Campylobacter* besmettingsroute voor pluimvee is een horizontale overdracht vanuit de omgeving. Het is aannemelijk dat *Campylobacter* vanuit de omgeving de stal wordt binnengebracht via de laarzen of ander gebruiksmateriaal van de dierverzorger. Varkens en pluimvee zelf (zowel vleeskuikens, leghennen als ouderdieren), en in mindere mate ook schapen en runderen zijn potentiële bronnen van *Campylobacter* besmetting.

Quinoloon behandeling is niet geschikt om een *Campylobacter* besmetting bij pluimvee uit te roeien.

Horizontale interventie maatregelen op bedrijfsniveau moeten nader bestudeerd worden en het effect van strikte toepassing van hygiëne maatregelen tijdens de gehele produktie periode zal op grotere schaal moeten worden geëvalueerd.

Résumé

Epidemiologie des *Campylobacter* en aviculture

Les campylobacters sont considerés comme une des causes principales des diarrhées humaines. On recense anuellement environ 300.000 cas de campylobacteriose aux Pays-Bas. En plus de la pathologue liée a l'infection, viennent s'ajouter des frais medicaux assez considerables et une perte importante de la productivité. Les produits d'origine avicole sont en grande partie tenus responsables de l'origine de l'infection chez l'homme. Une façon de lutter contre la campylobacteriose humaine et de diminuer sa frequence serait de reduire au minimum les sources de contamination des viandes de volailles. La manière la plus efficace et elegance pour atteindre cet objectif serait d'éviter la colonisation par *Campylobacter* des animaux vivants en période d'élevage. L'objectif de la présente thèse etait de chercher à avoir une meilleur idée sur l'épidemiologie de *Campylobacter* dans la filière avicole afin d'envisager des mesures efficaces permettant de réduire son importance.

Dans la plupart des études realisées dans le cadre de cette thèse, le système d'identification sérologique selon Penner a été utilisé comme marqueur épidemiologique. L'absorption d'antiserums et l'usage d'un système de groupage ont été introduit et les modifications apportées à la méthode originale figurent au Chapitre 2. Avec l'utilisation de ce système modifié, plus de 80% des *Campylobacter* isolés ont pu être sérotypés. En ce qui concerne les souches qui n'ont pu l'être, il s'agit de sérotypes qui jusqu'a présent n'ont pas été enregistrés dans le système utilisé. Le sérotypage répété d'un certain nombre de souches de *Campylobacter* ne suggère aucune instabilité des sérotypes au sein de ces souches. Toutefois la conservation des isolats de *Campylobacter* à -80°C avait une influence remarquable sur leur sérotypage ultérieur. Il est conseillé au cas où le sérotypage ne peut se faire immediatement, de conserver des extraits d'antigène provenant d'isolats frais de *Campylobacter* plutot que de préparer les antigènes à partir de cultures congelées.

Le Chapitre 3 décrit une étude effectuée sur des lots de reproductrices quant à la présence de *Campylobacter*. Dans neuf fermes avicoles possédant des reproductrices, soit au total 43 lots logés séparement, *Campylobacter* et *Salmonella* ont été recherchés. Ces travaux ont revolé que 29 des 43 lots etaient porteurs de

Campylobacter (67%) et 12 de *Salmonella* (28%). Deux des neufs fermes ne prévalont ni *Campylobacter* ni *Salmonella* au moment de prélèvements. Dans deux autres des *Campylobacters* ont pu être isoler mais pas des *Salmonelles*. Au total, 330 souches de *Campylobacter* ont été isolées puis serotypées. 19 sérotypes ont ainsi été identifiés. Des serotypes concordants ont été retrouvés au sein de tous les lots élevés dans une même ferme.

Le Chapitre 4 décrit une recherche effectuée sur le mode de contamination des pondeuses par *Campylobacter*. Deux groupes de pondeuses, élevées en batterie ou au sol, ont été suivies pendant leur periode de production. Dans les deux types de logement, une contamination des pondeuses par *Campylobacter* a été constatée. La seule différence résidait dans le fait que les ponseuses installées en batterie l'ont été plus tardivement que celles installées au sol. Le pourcentage d'isolats au sein des lots positifs au *Campylobacter* etait de 100% et les pondeuses ont continué à excréter le germe dans les fiente caecales jusqu'à l'abbatage. L'identification sérologique des *Campylobacter* laisse entrevoir une contamination horizontale à partir de l'environment. 179 oeufs provenant de lots de pondeuses excrétant le *Campylobacter* ont été examinés. Le germe a été isolé

Dans la fiente recueillie sur 3 de ces oeufs, a été identifié le germe du *Campylobacter*, mais pas dans leur jaune d'oeufs ou dans le jaune d'oeufs de 40 autres oeufs supplementaires examinés. Sur un total de 100 pondeuses examinées pour complement d'information, il s'est avéré que 93 pondeuses etaient porteuses de *Campylobacter* dans leur caecum. Le germe a été egalement retrouvé dans les ovaires (4x), la trompe (7x), l'oviducte (11x) et l'uterus (14x) de 21 sujets au total chez lesquels l'appareil reproducteur a été contaminé. Cette contamination provenait vraisemblablement d'une source faecale externe.

Quant au Chapitre 5, il décrit une étude longitudinale realisée sur deux entreprises avicoles aux Pays-Bas. Des lots de poulets de chair ont été examineés sur huit séances de production et ceci toutes les semaines sur la présence eventuelle de *Campylobacter* dans le cotenue des caecums. Des déchets de couvoir et la nouvelle litière ont été examiné avant le debut de chaque séance quant á la présence eventuelle du germe. L'eau, le fourrage, les insects et les excréments des autres animaux présent sur ces entreprises ont été échantillonnés. En général, la contamination des lots de poulets de chair se produisait entre trois et quattre semaines d'age et l'infection persiste jusqu'á l'abbatage. Le pourcentage d'isolat au sein des lots contaminés se situait autour des 100%. Au cours de chaque periode de

production, tous les serotypes de *Campylobacter* identifiés dans les differents poulaillers de ces entreprises, présentaient des patrons concordants. Et le prochain lot présentait en general un nouveau patron serotypiquement. La grande majorité des serotypes isolés chez les ponseuses, les porcs, les moutons et les bovins etaient differents de ceux identifiés au même moment chez les poulets de chair. Il est à signaler que les echantillons prelevés sur les déchets de couvoir, la nouvelle litière, l'eau et le fourrage n'ont montrés aucune trace d'existence de *Campylobacter*.

Le Chapitre 6 décrit une recherche effectué pour étudier le caractère saisonnier de la présence du *Campylobacter* et du *Salmonella* dans les prélèvements de caecum des poulets de chair sur les chaines d'abbatage. De mars 1992 à mars 1993, 187 lots de volailles provenant de trois différents abattoirs ont été au total examinés. Des échantillonages ont été faite sur un minimum de 10 lots toutes les quattre semaines et 25 caecums par lot ont été examinés. Sur 153 des 187 lots, *Campylobacter* a été isolé. La contamination des lots de poulets de chair par le *Campylobacter* presentait un caractère saisonnier, atteignant un niveau élevé (100%) entre les mois de juin et septembre et plus faible (50%) au mois de mars. La contamination par *Salmonella* ne subit pas cette influence saisonnière. Néanmoins, *Salmonella* a été identifié dans 49 lots sur un total de 181 lots examinés. On a pu par ailleurs établir une correlation positive entre la frequence de présence du *Campylobacter* et du *Salmonella* au sein des lots.

Le Chapitre 7 traite des résultats obtenus sur les tests de résistance aux antibiotiques et notamment aux quinolones. A l'aide de la méthode classique de tampons impregnés, 617 isolats de *Campylobacter* provenant de 150 lots de poulets, ont été testé quant à la sensibilité à la céphalotine (témoin), l'ampicilline, la tétracycline, l'érythromycine et aux quinolones comme l'acide nalidixique, la fluméquine, l'enrofloxacine, et la ciprofloxacine. On a pu constater une résistance croisée des isolats de *Campylobacter* aux quinolones utilisés. 181 isolats de *Campylobacter* (29%), provenant de 55 lots de poulets de chair (37%), se sont montrés résistant aux quinolones. De même, 94 isolats de *Salmonella* provenant de 40 lots ont été testés quant à leur sensibilité aux antibiotiques. Huit isolats (8,5%) provenant de trois lots (7,5%) s'etaient montrés résistant à l'acide nalidixique et à la fluméquine (ainsi qu'à la tétracyline) mais pas à l'enrofloxacine ou la ciprofloxacine.

Le Chapitre 8 décrit les résultats de la recherche sur l'induction de la résistance des souches de *Campylobacter* aux quinolones après traitement des poussins contaminés

par ca produit. Ce travail a porté sur six groupes de 15 poussins. A 19 jours d'age, on a administré à chaque groupe une dose de *Campylobacter jejuni* sensible aux quinolones. Les sujets recevent à partir du 26e jour d'age, et ceci pendant quattre jours durant, de la flumequine ou de l'enrofloxacine dans l'eau de boisson et à une concentration de 15 ou 50 ppm. Un des groupes de poussins a été traité auparavant avec de l'enrofloxacine pendant les quattre premiers jours de vie. Le traitement aux quinolones n'a pas éliminé la contamination des poussins par le *Campylobacter*. Aux jours 29, 33 et 43 des sujets provenant des deux groupes traités à l'enrofloxacine à partir de 26e jour d'age, il a été isolé des souches de *Campylobacter* résistants à l'acide nalidixique, à la fluméquine et à l'enrofloxacine. Tous les isolats de *Campylobacter* provenant de tous les autres groupes de poussins restaient sensible aux quinolones.

Enfin le Chapitre 9 content une discussion générale des resultats des diverses recherches effectuées. La figure 9.1 donne un aperçu sur les possibles voies de transmission du *Campylobacter* en rapport avec les resultats tels qu'ils sont présentés dans cette thèse.

Il n'a été trouvé aucune preuve de transmission verticale du *Campylobacter* des parents aux descendants, ni d'une transmission horizontale d'un lot à un autre à travers en poulailler.

La plus importante voie de contamination de la volaille est une transmission horizontale à porter de l'environment. Il est plausible que le germe se soit introduit dans les poulailers par les bottes et autres materials utilisés par les soigneurs. Les porcs et la volaille même (aussi bien les poulets de chair, les pondeuses comme les reproductrices) et dans une moindre mesure les moutons et les bovins sont de potentielles sources de contamination du *Campylobacter*.

Le traitement aux quinolones ne saurait être indiqué chez la volaille pour l'éradication du *Campylobacter*.

Une ensemble de mesures sur le plan horizontale au niveau des entreprises avicoles devra être étudié et les effets de l'application de strictes mesures d'hygiène sur toute la chaine de production devront être évalués.

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Curriculum Vitae

Wilma Froukje Jacobs-Reitsma werd op 29 oktober 1963 te Amsterdam geboren. In 1982 behaalde zij aan het Ichthus-College te Drachten het diploma Gymnasium-B. Later in dat jaar begon zij de studie Levensmiddelentechnologie aan de Landbouwhogeschool te Wageningen. In de doctoraalfase werden de hoofdvakken Gezondheidsleer en Levensmiddelenmicrobiologie gevolgd. De stageperiode werd doorgebracht bij de Rijkskeuringsdienst van Waren te Zutphen, waar zij in aansluiting op de stage van januari tot en met augustus 1988 werkte als laboratoriummedewerker. Tussentijds (maart 1988) studeerde zij af aan de Landbouwuniversiteit Wageningen. Najaar 1988 en voorjaar 1989 werkte zij, in opdracht van uitgeverij Noordervliet en in samenwerking met prof. dr. D.A.A. Mossel, aan het redigeren van de derde druk van het boek "Mikrobiologisch Onderzoek van Levensmiddelen, strategie, principes en methoden", door kenners ook wel "Het Blauwe Boek" genoemd. In juni van het gedenkwaardige jaar 1989 trad zij als onderzoeker microbiologie in dienst van het Centrum voor Onderzoek en Voorlichting voor de Pluimveehouderij "Het Spelderholt" te Beekbergen (tegenwoordig: "Vestiging Beekbergen" van het Instituut voor Veehouderij en Diergezondheid ID-DLO). Daar werkte zij de afgelopen jaren aan het onderzoek dat in dit proefschrift beschreven is.