Campylobacter in Denmark

– Control, human risk and source attribution

PhD Thesis
Louise Boysen
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DTU Food
National Food Institute

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Preface

The work presented in this thesis was carried out from March 2008 to January 2012 at The National Food Institute, Technical University of Denmark.

The PhD tasks were part of different research projects. The source attribution modelling work was part of the CAMSA (CAMpylobacter Source Attribution) project funded by the Danish AgriFish Agency (3304-FVFP-07-765-01). The studies on physical decontamination were part of a larger project entitled "Risk perception and cost benefit analysis of interventions to control *Campylobacter*", also funded by the Danish AgriFish Agency (FFS05-01). Evaluation of the Danish *Campylobacter* situation (2001-2010), including the evaluation of human risk from broiler meat, were activities under the program aiming at reducing *Salmonella* and *Campylobacter* in Danish and imported meat (2006-2010), which was funded by the Ministry of Food, Agriculture and Fisheries.

The Foundation Idella granted me a travel scholarship to support my attendance at the 16th international workshop on *Campylobacter*, *Helicobacter* and related organisms (CHRO), 28th of August to the 1st of September 2011, Vancouver, BC, Canada.

Supervisors of the project were Hanne Rosenquist (The Danish Zoonosis Centre at The National Food Institute, DTU), Tine Hald (the Epidemiology and Risk Modelling group at The National Food Institute, DTU), and Steen Ethelberg (Statens Serum Institut).

Søborg, January 2012
Louise Boysen

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Summary

This thesis provides an evaluation of the Danish *Campylobacter* situation from 2001 to 2010. The evaluation includes a description of the Danish situation in relation to the national control strategy and control measures applied at slaughter, an assessment of human risk of campylobacteriosis from retail broiler meat in the period 2001-2010 for assessment of the effect of implemented control measures, and finally, exploration of the apportioning of human campylobacteriosis cases caused by *Campylobacter jejuni* in Denmark to different sources of animal origin.

Danish action plans to control *Campylobacter* in broilers and broiler meat comprise initiatives covering the whole domestic food chain "from farm to fork". Initiatives involve biosecurity in the primary production; scheduling of *Campylobacter* negative flocks to production of chilled meat and positive flocks to the production of frozen products, to the extent possible, and consumer campaigns to reduce cross-contamination in domestic kitchens (manuscript I). In addition, a case-by-case control was introduced in 2007; targeting high-risk batches of fresh meat of Danish and imported origin. With the implementation of the first Danish initiatives and action plans against *Campylobacter*, the prevalence decreased in the Danish broilers from 2002 to 2004 as well as in chilled broiler meat at slaughter, 2004-2006 (manuscript I). The registered number of human cases was lower in the period after the implementation of action plans (2003-2010) compared to the period before (2001-2002).

The *Campylobacter* prevalence in Danish broiler flocks was found to be a strong predictor for the seasonal occurrence of *Campylobacter* in Danish chilled broiler meat. Seasonality was more distinct for chilled meat compared to frozen meat, and was more pronounced for Danish meat compared to imported meat (manuscript II).

The measures implemented in the major Danish slaughterhouses (forced air chilling, crust freezing and freezing) with the potential to reduce numbers of naturally occurring *Campylobacter* spp. were all shown to be effective to greater or lesser extent. Mean reductions of 0.44, 0.42, and 1.44 log units were obtained by forced air chilling, crust freezing, and freezing, respectively. Steam-ultrasound treatment using a proof-of-concept equipment resulted in a mean reduction of ≥2.51 log units. However, an adverse effect of this technique was a slightly boiled appearance of the carcass skin. Visceral rupture yielded an increase in *Campylobacter* of 0.9 log units and indicated that hygienic improvement of this process operation could lead to a reduction of nearly one log unit. It could be of interest, to investigate if a combined use of the methods would result in an additional reduction. No method performed equally to freezing when considering reductions in *Campylobacter* counts against adverse effects (manuscript III).

Despite extensive research into control measures applicable during slaughter, no intervention has yet been able to reduce *Campylobacter* contamination to negligible levels within legislation, without changing product characteristics (manuscript III). Further studies and development of hygienic and decontamination measures are needed. This area has been in focus for some time, and

substantial research has already been carried out. Therefore, a change in strategy may be needed; for example, more attention could be dedicated to the development of 'multiple hurdle' strategies.

With the exception of the log reductions obtained by freezing (for seven days) and treatment with TSP (marginally significant), log reductions obtained by decontamination, physical or chemical, in laboratory scale with inoculated meat medallions, tended to be unaffected by the initial *Campylobacter* concentration on the meat, for the decontamination techniques investigated. For these techniques, analyses suggested that different reductions were obtained using high inoculation levels (10^7 cfu/sample) compared to the lower levels (10^3-10^5 cfu/sample). All results were significantly influenced by strain. Accordingly, reductions obtained in studies with high concentrations of *C. jejuni* and one or few *C. jejuni* strains may not represent the general result for the species. For future studies, if investigations of naturally contaminated meat cannot replace inoculation studies, we advise to use a mixture of strains found in the production environment at levels as close to the natural contamination as possible (manuscript IV).

The human risk of campylobacteriosis associated with the four main categories of broiler meat available for sale at retail in Denmark (Danish and imported, chilled and frozen meat) was assessed by quantitative microbiological risk assessment. Human risk was generally higher for chilled meat compared to frozen meat. The most evident changes were; the decreasing risk from chilled imported meat (2005-2010) and the increasing risk from Danish frozen meat (2003-2010). No marked changes in human risk from Danish chilled broiler meat and imported frozen meat were observed. The reduction of the human risk from imported chilled meat coincides to great extent with the implementation of the case-by-case control. Accordingly, the case-by-case risk assessments may have provided the retailers with an incentive to heighten standards for their suppliers. The increasing risk from Danish produced, frozen broiler meat coincided with the control measure of scheduling of meat from *Campylobacter* positive broiler flocks to frozen production, to the extent possible. The allocation of meat from positive flocks results in a frozen production with a higher *Campylobacter* prevalence. Even though, the frozen meat is *Campylobacter* positive, it still constitutes a lower risk than chilled meat, as freezing reduces the *Campylobacter* numbers on the meat (manuscript V). The relative risk in total from broiler meat available for sale in Denmark increased from 2001 to 2005. This tendency was turned around after 2005 and remained on a stationary level with minor fluctuations. Changes in the relative risk occurred within the different categories of meat, but as they are counterbalancing each other the total risk was not markedly decreasing in the last part of the study period when compared to the first period of the study.

The use of QMRA in the evaluation of intervention strategies proved to be of added value, compared to using prevalence alone. The observed prevalence was not a very good surrogate measure for human risk. The approach of combining prevalence, mean concentration and also the variability of the mean concentration is important in relation to assessing the risk of human illness.

The source attribution models, the AI model and the CAMSA model, produced similar results based on MLST subtyping. Both models attributed the vast majority of cases (>50%) to the broiler chicken reservoir, while the second most important reservoir was cattle (mean estimate 16 and 17%,

the CAMSA model and AI model, respectively). Credibility intervals (95%) around the mean source estimates for the two models overlapped; hence, mean estimates for each source computed by the two models were not significantly different (manuscript VI).

Addition of the *flaA* gene to the MLST sequence type added slightly more discriminatory power between the broiler chicken and the cattle reservoir, apportioning a slightly higher proportion of cases to the broiler chicken reservoir at the expense of the cattle reservoir (manuscript VI).

The present PhD study has provided results which are useful for future risk management decisions regarding the control of *Campylobacter* in the broiler production, and in which reservoirs to target interventions.

Sammendrag (Summary in Danish)

Denne PhD afhandling beskriver og evaluerer den danske *Campylobacter* situation fra 2001 til 2010. Evalueringen inkluderer en beskrivelse af den danske situation relateret til den nationale bekæmpelsesstrategi og bekæmpelsestiltag implementeret på slagtelinjen, en vurdering af den humane risiko for campylobacteriosis fra kyllingekød solgt i detailforretninger i perioden 2001-2010 set i forhold til de implementerede bekæmpelsestiltag, og til sidst beskrives et smittekilderegnskab for kilder til humane *Campylobacter jejuni* tilfælde i Danmark baseret på fund af forskellige *Campylobacter* sub-typer i forskellige kilder af animalsk oprindelse.

De danske strategier til bekæmpelse af *Campylobacter* i slagtekyllinger blev iværksat fra slutningen af 1990'erne og fremad og inkluderer initiativer i hele produktionskæden "fra jord til bord". Initiativerne indbefatter biosikkerhed i primær produktionen, sortering af *Campylobacter* negative flokke til produktion af kølet kød og *Campylobacter* positive flokke til produktion af frosset kød i det omfang det er muligt, og iværksættelse af forbrugerkampagner om køkkenhygiejne for reduktion af krydskontaminering i private køkkener (manuscript I). I 2007 indførtes case-by-case kontrollen af dansk og importeret kød. Kontrollen er et supplement til de danske bekæmpelsesinitiativer og har til formål at reducere forekomsten af partier af kød med høj risiko for forbrugerne på grundlag af risikovurderinger af de enkelte partier. Analyse af overvågningsdata fra kyllingeflokke og kyllingekød fra perioden 2001-2010 viste, at *Campylobacter* prævalensen i kyllingeflokke faldt i perioden 2002 til 2004, og fra 2004 til 2006 sås et fald i *Campylobacter* prævalensen i det danske kølede kyllingekød (målt på slagteriet). Også forekomsten i det importerede kyllingekød faldt fra 2005 og frem (manuscript I). Antallet af registrerede humane cases var lavere i perioden efter implementeringen af bekæmpelsesstrategierne (2003-2010) sammenlignet med perioden før implementeringen (2001-2002).

Campylobacter prævalensen i slagtekyllingeflokke blev fundet til at være en stærk prædiktor for *Campylobacter* prævalensen i dansk kølet kyllingekød. Det betyder, at den sæsonvariation i prævalens, der findes for danske kyllingeflokke også var tydelig i det danske kølede kyllingekød. Sæsonvariation var mere udtalt for kølet end for frosset kød og var mest udtrykt for dansk kød, sammenlignet med importeret kød (manuscript II).

På de store danske slagterier er der indført processer, der potentielt kan reducere antallet af naturligt forekommende *Campylobacter* spp. (blæstkøling, skalfrysning samt frysning). Disse processer blev ved undersøgelser på slagteriet med naturligt kontaminerede flokke fundet at reducere antallet af *Campylobacter* i større eller mindre grad; med gennemsnitlige reduktioner på henholdsvis 0,4; 0,4; og 1,4 log enheder. Behandling med damp-ultralyd ved brug af et proof-of-concept udstyr, viste sig at give en gennemsnits reduktion på ≥2,5 log enheder. Dog havde overfladen af de damp-ultralyds behandlede kyllinger et let kogt udseende efter behandling. Brud på tarmsæt under tarmudtag gav en ekstra *Campylobacter* kontamination på gennemsnitligt 0,9 log enheder, hvilket indikerer, at optimeret hygiejne under denne proces ville kunne reducere antallet af *Campylobacter* på kødet med op mod en log enhed. Det kunne være interessant at undersøge om anvendelse af metoderne i kombination vil have en synergistisk effekt. Når man tager både

reduktionspotentiale og produktkarakteristika i betragtning var frysning den metode, der gav det bedste resultat (manuscript III). På trods af omfattende forskning indenfor dekontaminering af kød er der til dato ikke fundet nogen forbruger acceptabel metode, der kan fjerne *Campylobacter* fra kødet (manuscript III). Derfor synes det at være nødvendigt med yderligere studier indenfor produktionshygiejne og dekontaminering herunder studier af kombinationseffekter.

Reduktioner i antallet af *Campylobacter* opnået ved dekontaminering, fysisk eller kemisk, i laboratorieskala med podede kødprøver viste, at reduktioner opnået ved frysning i syv dage og behandling med TSP (marginal signifikant) var afhængige af startkoncentration af *Campylobacter* inden behandling. Ved høje startkoncentrationer (10^7 cfu/prøve) sås en større reduktion ved behandling end ved lavere startkoncentrationer (10^3-10^5 cfu/prøve). Ved frysning i 24 h og ved behandling med vinsyre var der ikke forskel på effekten ved forskellige startkoncentrationer. Derimod var der ved alle behandlinger en tydelig forskel i effekten mellem de anvendte *C. jejuni* stammer. Altså kan man ikke forvente, at reduktioner opnået i studier med brug af høj startkoncentration og en enkelt eller kun få stammer er repræsentative. Det anbefales, at man i fremtidige reduktionsstudier benytter naturligt kontaminerede prøver, alternativt prøver podet med et miks af stammer naturligt forekommende i kyllinger og i niveauer tilnærmet den naturlige forekomst (manuscript IV).

Den humane risiko for campylobacteriosis forbundet med kyllingekød tilgængeligt for salg i Danmark (dansk og importeret, kølet og frosset) i perioden 2001-2010 blev estimeret ved brug af kvantitativ risikomodellering (QMRA). Den humane risiko var generelt højere for kølet kød sammenlignet med frosset kød. De mest markante ændringer over tid var den faldende risiko fra importeret, kølet kød (2005-2010) samt den stigende risiko fra dansk, frosset kød (2003-2010). Ingen nævneværdige ændringer blev observeret for dansk produceret kølet kød samt for importeret frosset kød. Reduktionen i human risiko fra det importerede kølede kød falder sammen med implementeringen af case-by-case kontrollen. Den stigende risiko fra det dansk producerede frosne kød hænger sammen med indførelsen af sortering af kød fra *Campylobacter* positive flokke til frost produktion (manuscript V). Brugen af QMRA til evaluering af, om bekæmpelsesstrategierne har haft en effekt, viste sig at være af merværdi sammenlignet med at se på ændringer i prævalens i flokke og kød, som traditionelt har været gjort. Kombinationen af prævalens, gennemsnits koncentration og variation omkring den gennemsnitlige koncentration viste sig at være vigtig i forhold til vurderingen af risikoen for human sygdom. Den totale relative risiko fra kyllingekød tilgængeligt for salg i Danmark steg i perioden 2001 til 2005. Denne tendens blev vendt efter 2005 og forblev på et jævnt niveau med mindre udsving. Ændringer i den relative risiko forekom i de forskellige kategorier af kød, men disse ændringer udligner hinanden, hvorfor den generelle risiko fra kyllingekød ikke faldt i den sidste del af perioden, sammenlignet med den første periode før handlingsplanerne.

Modellerne, der blev anvendt til smittekilderegnskab for *C. jejuni*, AI modellen og CAMSA modellen, gav enslydende resultater baseret på sub-typning med MLST. Begge modeller tilskrev størstedelen af de humane tilfælde (>50%) til kyllingereservoiret, mens kvæg var det næst vigtigste reservoir (henholdsvis 16 og 17%, CAMSA og AI modellen). Konfidensintervaller (95%) for

estimaterne for de to modeller overlappede; altså er estimaterne fra de to modeller ikke signifikant forskellige (manuscript VI). Ved at anvende en kombination af *flaA* og MLST typer som grundlag for smittekilderegnskabet opnåedes en højere diskriminatorisk power. Dette var særligt udtrykt mellem kyllinge- og kvægreservoiret, og medførte en lidt højere andel af tilfælde der blev tilskrevet kyllingereservoiret på bekostning af kvægreservoiret (manuscript VI).

Dette PhD studie har leveret ny viden, som kan få stor betydning for fremtidige beslutninger vedrørende håndtering af *Campylobacter* i Danmark. Det gælder både for strategier i slagtekyllingeproduktionen, men også i relation til, om der skal indføres bekæmpelsestiltag for andre kilder.

List of abbreviations

AI model:	The Asymmetric Island model
AMOVA:	Analysis of Molecular Variance
C. jejuni:	*Campylobacter jejuni*
CAMSA:	CAMpylobacter Source Attribution
CFU:	Colony Forming Units
DANMAP:	Danish Integrated Antimicrobial Resistance Monitoring and Research Program
EC:	The European Commission
EU:	European Union
EFSA:	The European Food Safety Authority
FAO:	Food and Agriculture Organization
FSC:	Food Safety Criteria
GBS:	Guillain-Barré Syndrome
GHP:	Good Hygiene Practice
MC:	Microbiological Criteria
MLE:	Maximum Likelihood Estimation
MLST:	Multilocus Sequence Typing
PHC:	Process Hygiene Criteria
QMRA:	Quantitative Microbiological Risk Assessment
ReA:	Reactive arthritis
ST:	Sequence Type
TSP:	Tri-sodium Phosphate
UK:	United Kingdom
WHO:	World Health Organization

1. TABLE OF CONTENTS

2. OUTLINE

The present thesis includes an assessment of the Danish *Campylobacter* situation in the period 2001 to 2010, in which several initiatives were initiated to control the pathogen. The *Campylobacter* situation in Denmark is described and results of implemented *Campylobacter* strategies is assessed using risk assessment. Furthermore, the apportioning of human campylobacteriosis cases to different reservoirs of animal origin is explored.

The focus of the thesis is applied rather than methodical. Several different methods are used, but in depth work with the methodologies to obtain optimised applicability or development of new methods are not within the focus of this dissertation and therefore not explored further. For example, available models for quantitative microbiological risk assessment and source attribution are used.

The framework of the thesis is chronological with regard to the Danish *Campylobacter* situation. The topics of the thesis are firstly a description of the *Campylobacter* situation in Denmark with focus on broiler meat within the last decade. And secondly, a description of different possibilities to reduce numbers of *Campylobacter* on naturally contaminated broiler carcasses, using physical decontamination techniques at a Danish slaughterhouse. Thirdly, changes in occurrence of *Campylobacter* on broiler meat at retail over a period of ten years and the resulting human risk of illness hereof are assessed. Finally, the origin of *Campylobacter jejuni* found in human campylobacteriosis cases is explored by source attribution modelling using a microbial subtyping approach.

The thesis is divided in four main chapters:

- **The Danish *Campylobacter* situation (manuscript I and II)**
  This chapter describes the progression of the human health concern in relation to *Campylobacter*, changes in the *Campylobacter* occurrence in broiler flocks and broiler meat and the consequential risk management actions; involving surveillance programmes and development of control strategies towards this pathogen.

- **Interventions at slaughter (manuscript III and IV)**
  This chapter concerns interventions at slaughter with emphasis on physical decontamination techniques and description of own studies, which were carried out at a large Danish broiler slaughterhouse using naturally contaminated carcasses. The potential of the applied techniques to reduce the number of *Campylobacter* on fresh broiler meat is presented. Furthermore, the influence of contamination level towards the magnitude of reductions from decontamination is assessed based on laboratory experiments with artificially inoculated samples including four different inoculation levels and three different strains which were challenged individually by physical and chemical decontamination.

- **Evaluation of the implemented action plans (manuscript V)**
  The Danish strategy to control *Campylobacter* involves a number of initiatives including the voluntary action plans for the broiler production. This chapter includes an assessment of changes in human risk over a period of ten years from broiler meat available for consumption in Danish retail establishments; Danish meat versus imported meat. Based on relative risk estimates the effect of action plans is evaluated. National surveillance data were used as input in a quantitative risk assessment model including a consumer phase module estimating the risk of illness from different categories of meat from the years 2001 to 2010.

- **Source Attribution of human campylobacteriosis in Denmark (manuscript VI)**
  This chapter presents a Danish source attribution of human campylobacteriosis cases caused by *Campylobacter jejuni* to different putative sources of the pathogen. The source attribution was based on a microbial subtyping approach using MLST sequence types and *flaA* types of isolates collected in 2007 and 2008. Results from two different models are presented; the CAMSA model (modified after the Danish *Salmonella* model *(53)*) and the Asymmetric Island model *(122)*.

3. INTRODUCTION

Campylobacter has for several years been the leading cause of foodborne bacterial gastro infectious disease in the developed world. Cases are mainly sporadic. In Denmark, from 1992 to 2001, a distinct raise was observed from an incidence of 21.9 to 86.4 per 100,000 population. From 2001 to 2003 the incidence dropped to 65.7 and has been steady since with minor fluctuations. The vast majority of cases is caused by *Campylobacter jejuni* (96% in 1997 *(90)*). About one third of cases are acquired by travelling abroad *(35)*. Incidences are based on the number of registered cases. However, as symptoms in most cases are not extremely severe, a proportion of cases are not necessarily seeking help at their general practitioner or at the hospital, resulting in underreporting of the disease. The actual number of cases ill from *Campylobacter* in Denmark was considered to be approximately four times higher than the registered number of cases *(33)*. Little is known about the role of immunity. However, the small amount of available research suggests that continuous exposure to heterologous *Campylobacter* strains may induce protective immunity *(60)*.

The most frequent symptoms of illness induced by *Campylobacter* infection are diarrhoea (often bloody), nausea, vomiting, fever, headache, abdominal and muscle pain. The majority of cases are self-limiting within 3-10 days without treatment. However, campylobacteriosis can be severe and life-threatening. In rare cases, chronic sequelae such as the Guillain-Barré syndrome (GBS) and Reactive arthritis (ReA) (formerly referred to as Reiter syndrome) and even death may occur *(13, 14)*. Both GBS and ReA are thought to be autoimmune responses stimulated by infection. Symptoms of GBS are acute neuromuscular paralysis, damaging the nerves of the peripheral nervous system. GBS have been reported to occur in 1-2 campylobacteriosis cases per 100,000 *(67)*. The classical syndrome of ReA involves a group of inflammatory symptoms (urethritis, conjunctivitis, and arthritis) *(24)*, however, the majority of cases do not present all symptoms. An incidence of 4.3 cases per 100,000 have been reported for ReA associated with *Campylobacter* infection *(57)*. *Campylobacter* infections rarely result in long term and/or disabling sequelae or even have a fatal outcome *(62, 63)*. However, the large numbers of cases affected by infection still result in great socioeconomic expenses due to days away from work, potential visit to the general practitioner and non-diagnosed sequelae *(71)*. Young, old, pregnant and immune-compromised groups are more often subject to illness requiring hospitalization and resulting in death compared to other groups *(48, 63)*.

The *Campylobacter* organism is according to literature fairly easy to kill. *Campylobacter* has been shown to be sensitive to desiccation *(31)*, heat *(28, 114, 119)*, freezing *(47, 107)*, acids *(21, 25, 26)*, UV radiation *(68)*, etc. However, even though *Campylobacter* is fairly easy to kill, humans are still getting ill from the bacterium. The infectious dose is believed to be low. Furthermore, different matrices, e.g. various foods, might pose as protective vehicles for the bacteria. *Campylobacter* do not grow below 30 °C *(61)* and the possibility of the bacteria to multiply outside a host in Denmark is regarded unlikely. Though, *Campylobacter* survives remarkably well at chill temperatures (around 4-5 °C) *(20, 74, 113)*. *Campylobacter* numbers decrease when stored at both 25 °C and -25 °C, however, at 4 °C numbers are basically unaffected. Observations have been reported to be similar with no influence of atmosphere (micro aerobic versus aerobic), strain or matrix (laboratory

media versus foods) *(20, 55, 56, 72, 74, 113)*. Cross-contamination with *Campylobacter* is considered to be very important in relation to exposure from various foodstuffs.

The risk pathways for human exposure to *Campylobacter* are many. *Campylobacter* spp. are found widespread in the environment and in farmed animals *(11, 42)*. Multiple sources of *Campylobacter* have been recognized, with different importance in relation to human illness. However, poultry, and especially broilers and meat thereof, has been recognized as the most important single source in Denmark, as well as in many other countries *(41, 81, 122, 123)*. *Campylobacter* is transferred to foodstuffs of animal origin during slaughter, while contamination of fruits and vegetables are believed to most often be related to irrigation, fertilization and handling during harvest, processing and handling of the produce *(19, 36)*. *Campylobacter* contamination of broiler carcasses during slaughter is primarily due to cross-contamination during the feather removal and evisceration process *(105)*. *Campylobacter* is mainly considered to be a surface contamination problem *(77)*.

Many countries have established monitoring programs towards *Campylobacter* in the broiler production, though the construction of these programs including sampling and testing scheme varies between countries and data are not directly comparable. To establish a baseline and comparable values for all Member States within the EU, a harmonized baseline survey was carried out in 2008, to determine the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses at slaughterhouse level *(39)*. In brief, this study showed great variation in *Campylobacter* prevalence for broiler carcasses (5-100%). The *Campylobacter* counts on broiler carcasses also varied widely between countries and between slaughterhouses within countries. In general, there was a tendency for high counts in countries with high *Campylobacter* prevalence *(39, 40)*.

Several risk assessments on *Campylobacter* in broiler meat has been developed within the last ten years. The risk assessments are used for different purposes; i.e. to evaluate potential effects of control measures in the broiler production chain and to assess the human risk due to *Campylobacter* in broiler meat *(86)*. Different scenarios have been assessed with regard to human risk reduction. FAO/WHO investigated the effect of change in prevalence and change in level of contamination *(45)*. Change in prevalence, or frequency with which chickens are contaminated at the retail stage will correspond to a proportional change in risk; e.g. a 50% reduction in prevalence is estimated to result in a 50% reduction in expected risk. Change in contamination level, corresponding to a reduction of concentration at retail level below 2.5 log units would considerably reduce human risk (non-linear relationship). Others investigated the effect of implementation of control measures in the primary production versus at the slaughter house. For example, the Danish quantitative microbiological risk assessment (QMRA) inferred that the human incidence could be reduced 30 times by introducing a 2 log reduction of the number of *Campylobacter* on chicken carcasses or reducing flock prevalence 30 times *(104)*. It should be noted that these evaluations of efficacy specifically concerns the reduction of risk for the fraction of human cases associated with broilers and broiler meat, and not human campylobacteriosis cases as a whole. Interventions implemented at reservoir level should not be neglected as they may potentially affect several transmission routes

whereas interventions later in the production chain will be restricted to the transmission route in question. Furthermore, it should be stressed that one strategy does not rule out the other.

The importance of *Campylobacter* concentration at the point of consumption in relation to risk of illness has been emphasized by several QMRAs which also point at the most effective intervention measure is reduction of the *Campylobacter* concentration, rather than reducing the prevalence *(86)*.

Several decontamination techniques to reduce the number of *Campylobacter* on broiler meat of chemical as well as physical character have been reported. Different chemical compounds have shown potential for *Campylobacter* reduction; e.g. acetic acid, acidified sodium chlorite, chlorine, tri-sodium phosphate, etc. *(75, 93, 112, 124)*. Use of chemical decontamination is a valid control measure in some countries (e.g. New Zealand and USA). In the European Union (EU), the legal basis for using authorized chemical substances[1] as a processing aid to remove surface contamination on poultry meat, has been effective from January 2006. Several chemical substances have been put up for evaluation, but due to inadequate scientific documentation, none have been approved yet *(33)*[2]. Studies of various physical decontamination techniques to reduce *Campylobacter* concentration have been published; e.g. concerning freezing, hot water, steam, irradiation, etc. *(69, 99, 107, 121)*. Physical decontamination does not need approval by authorities (except irradiation). Freezing and irradiation seem to be the most effective techniques to reduce *Campylobacter* concentration. However, irradiation is not authorized for use in all EU countries[3] (including Denmark) and the consumer acceptance is limited. Further, the process of freezing changes the product characteristics, i.e. resulting in products which cannot satisfy the consumer demand of chilled meat. Each technique has its advantages and disadvantages in relation to product appearance, consumer acceptance, costs, etc. To date, no single decontamination method has been established as the solution to solve the *Campylobacter* problem.

Multiple studies on reduction of *Campylobacter* on broiler meat by decontamination at slaughter have used artificial inoculated samples; though, the influence of different inoculation levels on reductions is rarely included in the investigations. Several of the studies are using high inoculation levels (10^7-10^8 cfu) as oppose to levels emulating what is found on naturally contaminated samples *(29, 34, 94, 100, 124)*. This issue may be of great importance in evaluation of techniques, if the magnitude of reductions is influenced by the initial concentration level. Furthermore, strain variation has not been thoroughly described. Most studies use a single strain or multiple strains in a mixture, thus prohibiting the possibility to assess the strain variation. A few studies, however, regarded this issue reporting different results depending on study/decontamination technique *(21, 93, 94)*. If the magnitude of reduction is influenced by inoculation level and strain

[1] Substances require prior authorization. The European Food Safety Authority performs the safety and efficacy evaluation of substance applications and the European Commission is responsible for the final approval.
[2] For additional information is referred to the EFSA Decontamination of carcasses homepage: http://www.efsa.europa.eu/en/topics/topic/decontamination.htm?wtrl=01
[3] The document "List of Member States' authorisations of food and food ingredients which may be treated with ionising radiation" can be retrieved from the homepage: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:C:2009:283:0005:0005:EN:PDF

variation, this may cause unintended bias in assessment the reduction potential of a given decontamination technique.

Action plans against *Campylobacter* have been implemented in several countries; Denmark, Norway, Iceland, the United Kingdom (UK) and New Zealand *(9, 10, 66, 103, 110, 117)* (and manuscript I). A schematic illustration is presented in Appendix table 1. The action plans all target the domestic broiler production. The two main focus areas of the control strategies are the same; on-farm biosecurity to reduce broiler colonization and reduction of *Campylobacter* numbers on broiler meat to reduce consumer exposure to the pathogen. The means for reducing *Campylobacter* numbers at slaughter varies between countries. In brief, meat from positive flocks was mandated to be frozen in Iceland and Norway (in Norway, heat treatment is an alternative to freezing). In Denmark, meat from positive flocks should be frozen, to the extent possible (voluntary strategy, but adopted by the large slaughter companies). In the UK and New Zealand, the choice of means to reduce *Campylobacter* numbers has been left to the industry.

Performance targets for *Campylobacter* have been adopted in a few countries; e.g. New Zealand, and the UK. Targets may concern a specific reduction of cases and/or processing criteria at production level. In New Zealand, mandatory targets have been set for broiler carcasses at the end of processing (on exit from immersion chiller) based on enumerative levels (average carcass count of 3.78 log cfu/carcass[4]), with escalating regulatory responses if targets are not met *(91, 110)*. In the UK, a new voluntary target has recently been set for UK produced raw chicken, as part of the newly developed *Campylobacter* Risk Management Programme. This is a joint action plan on *Campylobacter*, which has been agreed upon by between FSA, Defra, British Poultry Council, National Farmers Union and British Retail Consortium on a voluntary basis. The target is to be reached by April 2015. The aim of the target is to reduce levels of the most contaminated chickens at the end of the slaughter process (post-chill). The target focuses on decreasing the proportion of birds in the most contaminated group (>1000 cfu/g). In 2008 the proportion was 28%. The aim is to reach 19% in 2013 and 10% in 2015 *(9, 10)*.

The domestic exposure of humans to *Campylobacter* involves various transmission routes; foodstuffs, drinking water, animal contact, outdoor leisure, and occupation *(41)*. Even though the general belief is that broiler meat is the largest single source of human infection, this still only accounts for a fraction of the cases. Several tools to investigate the potential sources of human disease are available; comparative exposure assessment, microbial subtyping, case-control studies, and analysis of outbreak data. Disclosure of the sources of human infections is crucial to develop targeted control strategies for campylobacteriosis. Two examples of *Campylobacter* source attribution of domestically acquired cases have been performed in New Zealand *(81)* and in the UK *(122)*, both using the microbial subtyping approach.

In spite of the extensive work carried out in relation to the control of *Campylobacter* in Denmark, no comprehensive evaluation of efficacy and effects has been performed, enabling an assessment of the work. Furthermore, efforts to track the sources of sporadic human cases in a

[4] Moving window method with a high count limit of 5.88 log cfu/carcass, detailed description in reference *(91)*.

broader perspective than identification of specific risk factors; e.g. investigating if consumption of broiler meat cause an elevated risk for campylobacteriosis, have not been considered. The present PhD study undertake the evaluation of results of the established action plans against *Campylobacter* in Denmark; focussing on the control of this pathogen on broiler meat, the human risk from broiler meat and disclosure of sources associated with the registered human cases (sporadic and domestically acquired).

4. OBJECTIVES

Campylobacter spp. continues to be a human health problem, causing society great socioeconomic costs. Extensive work has been performed around the world trying to manage different aspects of the problem; including studies of various control measures, risk assessment, source attribution, etc. The aim of this thesis was to assess the Danish *Campylobacter* situation by evaluation of control measures, changes in human risk and source attribution. The main objectives were

- To evaluate the development in the *Campylobacter* situation, in particular within the period 2001 to 2010, in relation to human cases, broilers and broiler meat (with emphasis on a detailed assessment of the retail broiler meat) and to describe existing control strategies.

- To explore the reduction in numbers of naturally occurring *Campylobacter* spp. on broiler meat at slaughter of selected physical decontamination techniques; freezing, crust freezing, forced air chilling and steam-ultrasound. And to investigate the influence of initial concentration and strain on the magnitude of reductions obtained by decontamination (physical and chemical).

- To evaluate the influence of the implemented strategy against *Campylobacter*, with emphasis on the first action plan, initiated in 2003, by assessing if the Danish action plans have had an effect directly on the human risk from retail broiler meat available for consumption in Denmark.

- To explore the apportioning of human campylobacteriosis cases caused by *Campylobacter jejuni* in Denmark to different sources of animal origin; using two existing source attribution models by means of the microbial subtyping approach based on MLST sequence types, *flaA* types, and antimicrobial susceptibility of isolates collected in 2007 and 2008.

5. THE DANISH CAMPYLOBACTER SITUATION (manuscript I and II)

5.1 Human incidence

In Denmark, human campylobacteriosis has been notifiable since 1980. The number of human campylobacteriosis cases increased rapidly from 1992 to 2001 and surpassed human salmonellosis cases in 1999 to become the most frequent cause of disease from zoonotic bacteria. An illustration of the registered number of human cases for the period of the study is presented in (Figure 1). Confidence intervals within years are based on a Poison distribution[5]. The 95% CI of number of cases is presented in Figure 1. Based on the confidence intervals the level of human cases was higher in 2001 and 2002 compared to the periods 2003-2007 and 2008-2010, where action plans have been implemented. The exact numbers of registered human cases and differences between years can be seen in Appendix table 3.

About one third of the human cases are estimated to be related to travel *(35)*. The estimate is based on telephone interviews of a part of the diagnosed campylobacteriosis cases from three Danish regions. This way of acquiring travel information was initiated in 2007. Due to the nature of data, the variation between years and seasons cannot readily be evaluated.

The incidence for different age groups (2009) is depicted in Figure 2. The incidence is particularly high for toddlers (1-4 years of age) and young people within the age group 15-34 years of age. Similar distribution of incidence between age groups was observed in the year 2000 *(2)*, indicating that this is not markedly varying. Comparable high incidence for children below four years of age is observed within the EU, however, the incidence for young people (age group 15-24) was markedly lower *(42)*. Young people have been identified to be of higher risk as a result of being less experienced in hygienic food handling *(15, 104)*.

[5] Viewing the whole Danish population every year as a sample it represents the state of the Danish population at one point in time. Hence it is a sample of possible states of the Danish population related to the year specific risk of getting campylobacteriosis. Under this assumption, the sampling error connected to the number of reported cases of campylobacteriosis can be estimated using a Poisson distribution assuming a constant population size over time.

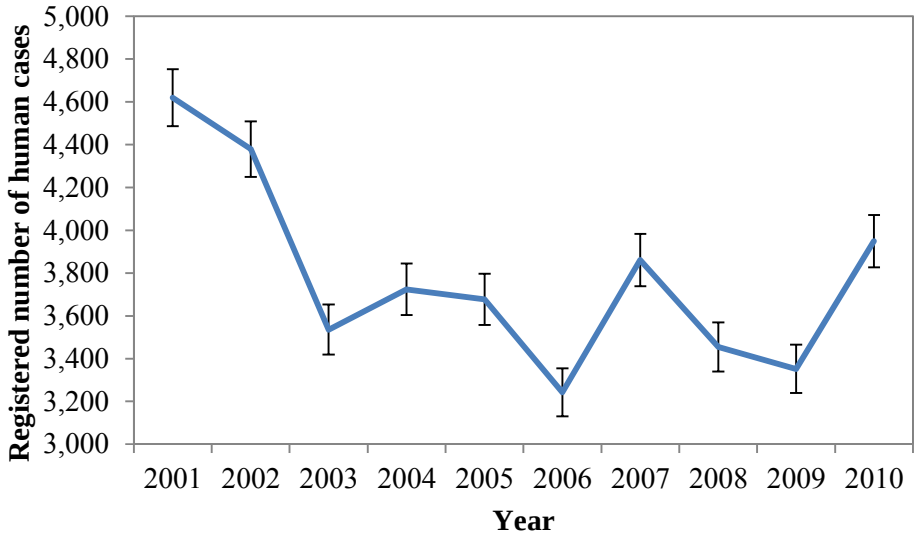

Figure 1. The registered number of human cases in Denmark 2001-2010 *(12)*, including 95% confidence intervals.

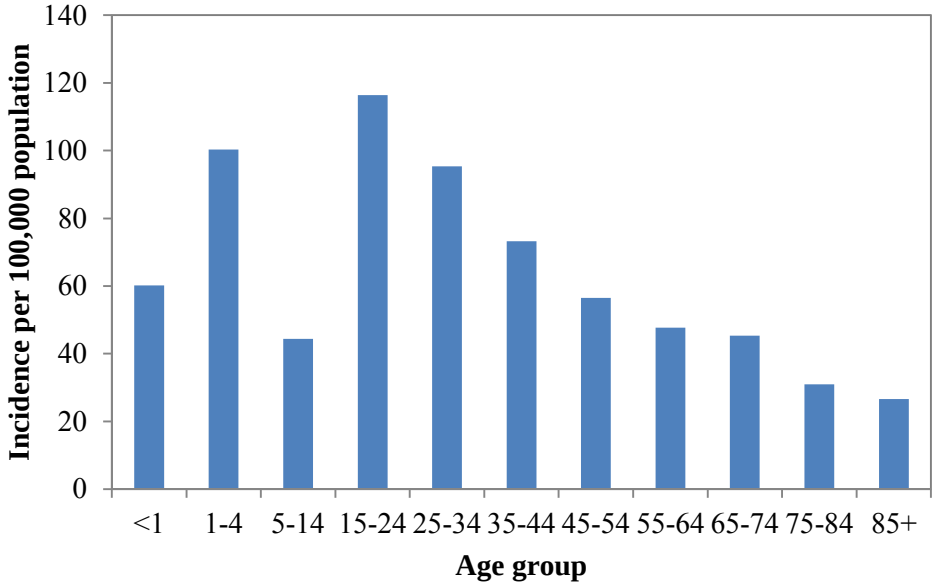

Figure 2. Incidence of campylobacteriosis for different age groups (2009 data) *(12)*.

Adjustment for the seasonality in human campylobacteriosis was performed as the seasonality in theory may mask variations. This was performed by a seasonal decomposition of the registered number of human campylobacteriosis cases on a monthly basis. The deseasonalised trend (Figure 3) is very similar to the observed data (Figure 1) and did not reveal unrecognized fluctuation.

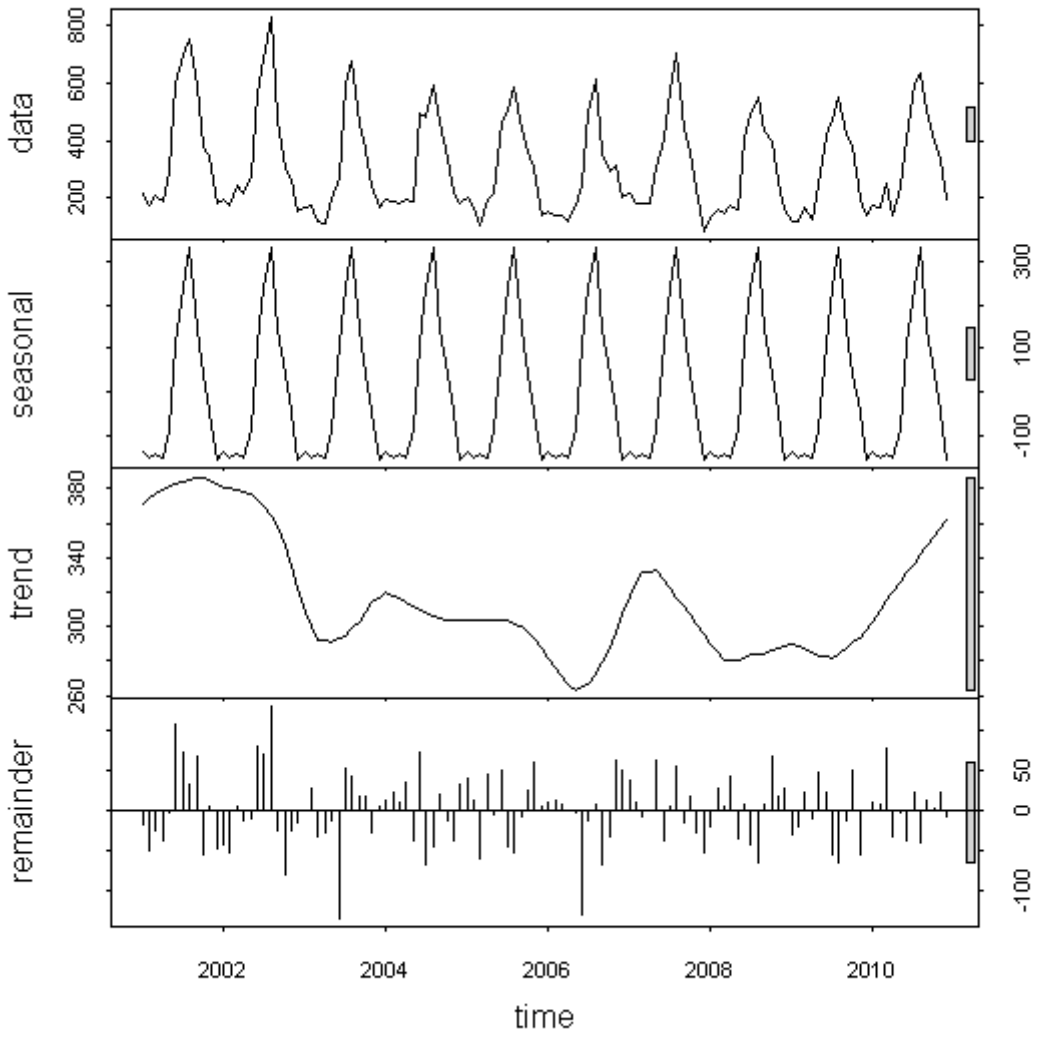

Figure 3.Seasonal decomposition of the monthly registered number of human cases, 2001-2010; comprising from the top down the observed data on a monthly basis, the seasonal component, the deseasonalised trend, and the residual. The unit on the vertical axis is the number of human cases.

5.2 Risk management including action plans

The first initiatives to control *Campylobacter* in Denmark were launched in 1995 targeting the primary production of broilers. Furthermore, data collection was initiated by the establishment of surveillance programmes (section 5.2.2). In 1998, a risk profile was developed; recommending development of a formal risk assessment for *Campylobacter jejuni* in foods and water *(1)*. However, a great lack of data on *Campylobacter* in water and foods other than broiler meat made the task very difficult. Consequently, a QMRA of *Campylobacter* in broilers and broiler meat was published in 2001 *(27)*. The main messages of the risk assessment work were a comparison of the potential to reduce human risk by application of interventions in a chain perspective "from farm to fork", and

the identification of the age group at highest risk of becoming ill (18-29 year olds). It was inferred that introduction of control measures to reduce the number of *Campylobacter* on broiler carcasses by a 2-log reduction would be an efficient and feasible management option; however, with recognition of the importance of implementing control measures throughout the chain to obtain the best possible synergetic effect. Based on the conclusions of the risk assessment, the first Danish action plan against *Campylobacter* in broilers and broiler meat was drafted and implemented in 2003 (section 5.2.1).

5.2.1 Danish action plans

The first action plan, which was introduced in 2003, comprised; control strategies concerning biosecurity in the primary production; scheduling of *Campylobacter* negative flocks to production of chilled meat and positive flocks to the production of frozen products, to the extent possible, and consumer campaigns to reduce cross-contamination in domestic kitchens (manuscript I). Thus, initiatives covered the whole domestic chain "from farm to fork". However, the Danish retail marked comprises both domestically produced products as well as imported products. Therefore, in addition to the above mentioned initiatives, came the case-by-case control in 2007. This initiative aimed at reducing high-risk batches of fresh meat from both Danish and imported meat. In brief, a number of imported and Danish batches of fresh meat are examined for numbers of *Campylobacter*[6] and based on these results the relative risk from a batch is assessed using quantitative microbiological risk modelling. If a batch is considered injurious according to article 14 in the EU food law (Regulation (EC) 178/2002), the food producing establishments cannot market the batch and marketed batches must be withdrawn *(6)*.

In 2008, a new four-year action plan was adopted with the aim to further decrease the prevalence and the concentration of *Campylobacter* in broilers and broiler meat. Generally, the new plan intensified the initiatives already in place. The most innovative initiative in the new action plan is the intention of developing applicable fly screens for broiler houses.

5.2.2 Surveillance

Surveillance of *Campylobacter* in various sources has been carried out during different periods. Broilers (all flocks) and broiler meat (at retail) have been the most frequently sampled sources, with surveillance programmes that were implemented and have run continuously since 1998 and 1995, respectively. Prior to 1998, a smaller number of broilers were monitored in the DANMAP project[7]. Furthermore, in 2004, *Campylobacter* surveillance of chilled broiler meat was established at the two largest slaughterhouses in Denmark; comprising approximately 98% of the Danish production of chilled broiler meat.

[6] Samples are also examined for the presence of *Salmonella* as case-by-case control is also in place for this organism.
[7] DANMAP is the Danish programme for surveillance of antimicrobial resistance in bacteria from livestock, food, and humans.

Other animal sources of *Campylobacter* (primarily cattle and pigs), have also been monitored via the DANMAP project since 1995. Different food sources have been investigated within different surveillance projects (CCL projects[8]) comprising different years (Appendix table 2). Results from the surveillance have confirmed poultry meat to be the source most frequently contaminated with *Campylobacter*.

The distribution of *Campylobacter* spp. varies between sources. The species most frequently associated with human disease is *C. jejuni*. This is also the species most frequently isolated from poultry and cattle, while for pigs the most commonly isolated species is *Campylobacter coli*.

Different schemes of analysis have been used why results from different surveillance projects are not directly comparable. While the detection of *Campylobacter* in broilers is carried out by using qualitative analyses (PCR) (described by Lund *et al. (78)*), the surveillance of *Campylobacter* in chilled broiler meat at slaughter is carried out by quantitative analyses with a detection limit of 10 cfu/g (described by Rosenquist *et al. (105)*) and in broiler meat at retail the analyses are performed by means of semi-quantitative analyses with a detection limit of 0.1 cfu/g *(5)*.

5.2.2.1 Data

The assessment of the *Campylobacter* situation (section 5.1 and 5.3) is based on the national surveillance data from 2001 to 2010.

- Data on the registered number of human cases was obtained from Statens Serum Institut, from the module of public health surveillance (http://www.ssi.dk/Smitteberedskab/Sygdomsovervaagning.aspx)
- Data regarding the *Campylobacter* occurrence in broiler flocks was obtained from the national surveillance (all flocks sampled)
- Data regarding the *Campylobacter* occurrence in meat was obtained from the national surveillance (random sampling)

5.3 *Campylobacter* prevalence in farmed animals and food (2001-2010)

For production animals (broiler flocks, pigs and cattle), the occurrence trends for *Campylobacter* differed in the period 2001-2010. The occurrence in broilers decreased from 2002 to 2004 from around 40% to around 30%, hereafter the prevalence remained steady at this level (Figure 4) (exact numbers can be seen in Appendix table 3). The occurrence in cattle did not change markedly (fluctuating around 60 to 70% in the period 2001-2010), while in pigs the prevalence decreased from 93 to 56%, with fluctuations, in the period 2003-2009.

In chilled meat from pigs and cattle, the prevalence was estimated to be 0.2 and 0.1%, respectively[9]. In retail broiler meat the prevalence differed between different categories of meat;

[8] Centrally Coordinated Laboratory projects (CCL projects) are national projects or surveys carried for a number of different purposes, e.g. monitoring and collection of data regarding foodborne hazards *(11)*.
[9] Surveillance data from 2002; investigation of non-heat treated retail samples of pork (N=2,413) and beef (N=3,046).

Danish and imported, chilled and frozen meat (Figure 5 and Appendix table 3). The prevalence in imported meat was in general higher than in Danish produced meat, and *Campylobacter* was more prevalent in chilled compared to frozen meat. These differences, however, diminished over years. *Campylobacter* occurrence is influenced by both the origin of the meat (Danish vs. imported) and by chilling method (chilled vs. frozen). From 2001 to 2007, a decreasing trend was observed for the prevalence in imported chilled meat, while an increasing trend was observed for frozen meat. For Danish chilled meat, neither increasing nor decreasing trends were observed for this period (from trend analysis, manuscript II). Data on concentrations were not regarded in this section, but are presented in manuscript V.

Other types of poultry meat available for sale in larger quantities are chilled turkey meat and frozen duck meat. The prevalence of *Campylobacter* in these meat categories fluctuates around 50% (2008-2010).

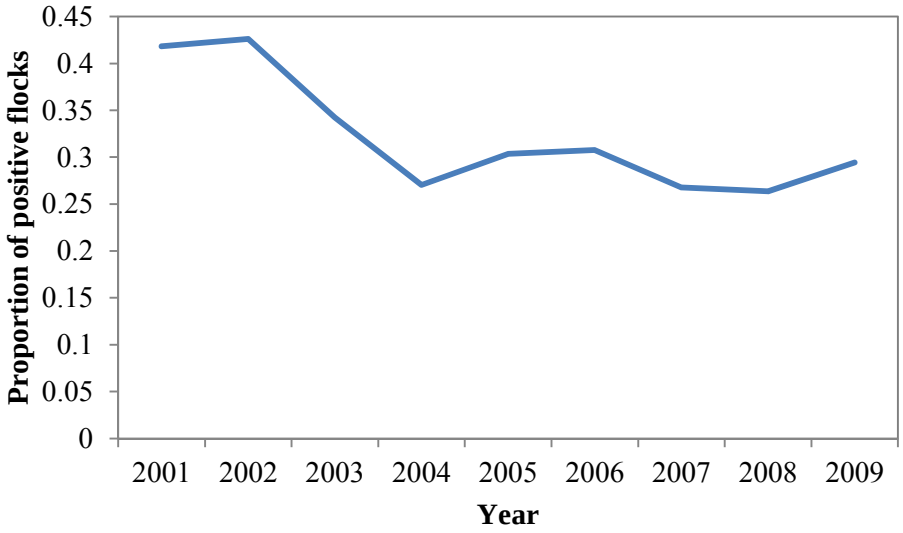

Figure 4. Prevalence of *Campylobacter* spp. in Danish produced broiler flocks, 2001-2009[10].

[10] No confidence limits are applied as it is the entire population of Danish broiler flocks that have been tested. The year 2010 was not included as the analysis method was changed.

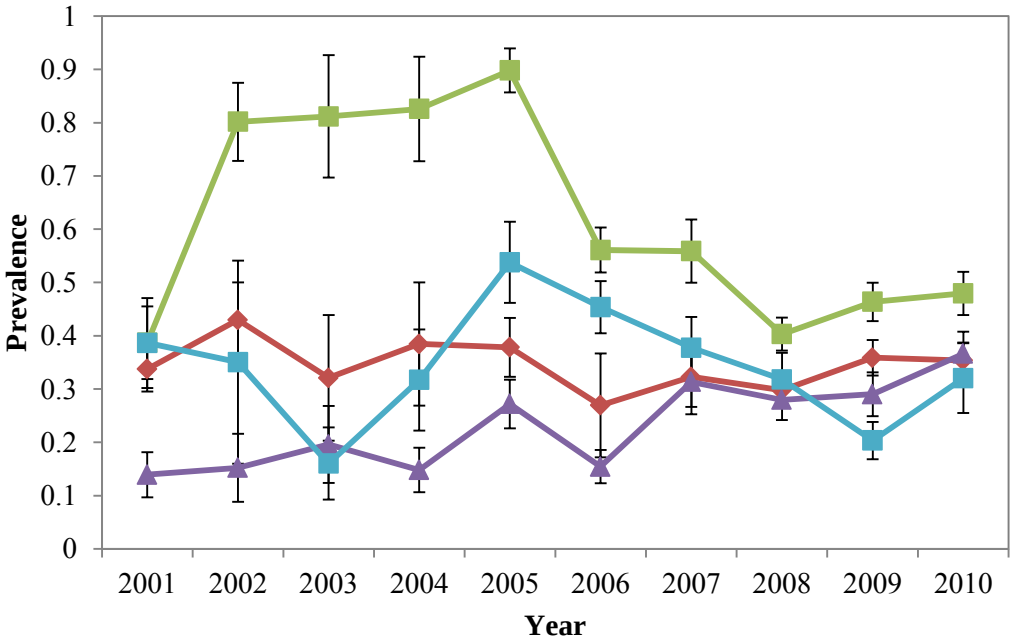

Figure 5. Observed prevalence (mean of quarters) in retail broiler meat (2001-2010). Danish chilled meat (blue), Danish frozen meat (red), imported chilled meat (green), imported frozen (purple) (national surveillance data), including 95% confidence intervals.

5.3.1 *Campylobacter* seasonality in broilers and broiler meat (manuscript II)

In Denmark, the incidence of human campylobacteriosis cases, as well as the *Campylobacter* prevalence in broiler flocks, is coincidently influenced by season with a summer peak in July/August (Figure 6).

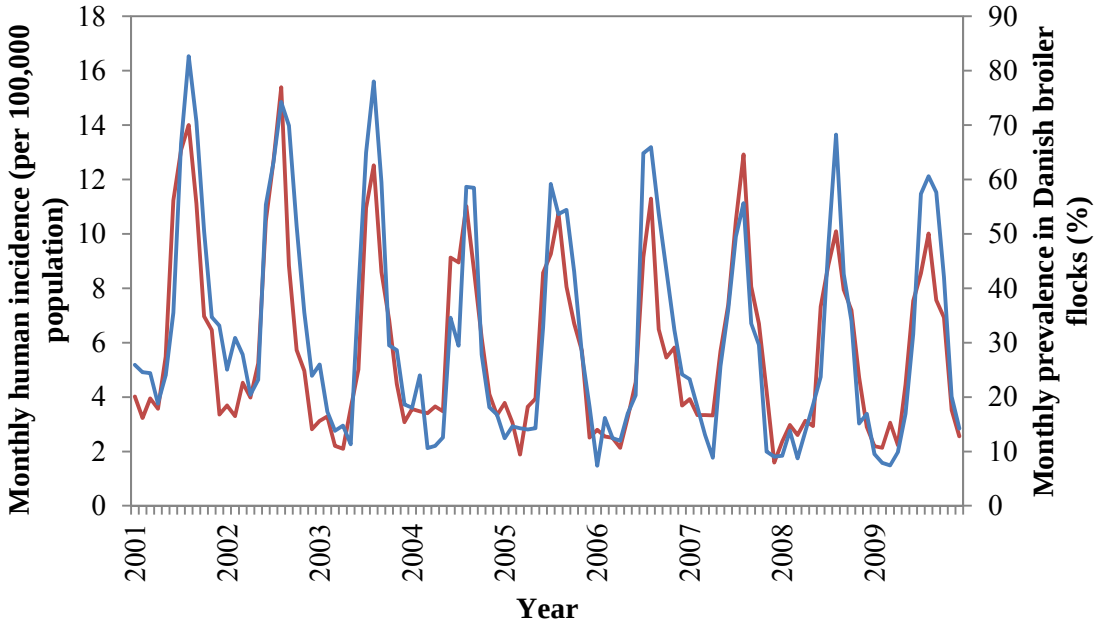

Figure 6. Seasonality of human campylobacteriosis incidence and *Campylobacter* prevalence in broilers. Monthly human incidence per 100,000 population (red), monthly prevalence in Danish broiler flocks (%) (blue) (national surveillance data).

A distinct seasonality of *Campylobacter* is also observed in chilled broiler meat at the large Danish slaughterhouses. The *Campylobacter* prevalence decreased markedly within the period 2004-2006 from 18% to 8% (manuscript I).

The occurrence of *Campylobacter* in broiler meat at retail has also been shown to be influenced by season, being more prevalent over the summer and fall. These observations were more distinct for chilled meat compared to frozen meat, and were most pronounced for Danish meat compared to imported meat. Trends in the *Campylobacter* prevalence for broilers and broiler meat in the high and low prevalence periods were similar (manuscript II). Thus, no factors or circumstances seem to have had a specific seasonal influence; i.e. decreasing prevalence in the warm or cold period individually.

Campylobacter prevalence in Danish broiler flocks was found to be a strong predictor for the occurrence of *Campylobacter* in Danish chilled broiler meat. However, besides flock prevalence, there was also a direct effect of season on the occurrence of *Campylobacter* which is not readily explained (manuscript II). Several factors may be of importance, but still have to be investigated. Such factors include; high within flock prevalence, high concentrations of *Campylobacter* in the gut contents, high degree of compliance with the scheduling strategy, use of substitute workers during vacation periods, etc. If these factors could be identified and were factors that could be acted upon, it could allow for implementation of more cost effective management strategies.

5.4 Discussion

The management of *Campylobacter* has been a "work in progress" and the methods used for *Campylobacter* surveillance in broilers and broiler meat have changed over time, why results from different time periods may not be directly comparable. The methods applied, generally reflect the newest and best methods available at the time they were launched. The newest enumeration methods, rather than comparability between investigations (using the same method) have been prioritized. There has been no optimal point in time to launch a standardized method for *Campylobacter* enumeration, because shifts in methods would degrade the possibility to follow up on the effects of the implemented interventions. However, the effects of the action plans have now been evaluated. Hence, methods for enumeration will be standardized for *Campylobacter* in meat from 2012.

With the implementation of the first Danish initiatives and action plans against *Campylobacter*, the prevalence decreased in the Danish broilers from 2002 to 2004 as well as in chilled broiler meat at slaughter, 2004-2006. Surveillance at the slaughterhouse has only been ongoing since 2004, which is why the prevalence of *Campylobacter* in broiler meat cannot be evaluated in the years before. No distinct decrease occurred in the *Campylobacter* prevalence in broilers from 2004 to 2006, indicating that factors other than the prevalence in broilers may be responsible for the decrease observed in meat sampled at the slaughterhouse. Such factors could include effective scheduling of meat from positive flocks to the production of frozen products and/or other initiatives implemented during processing to bring down *Campylobacter* concentration; e.g. by crust freezing and forced air chilling (manuscript III). Lowering the concentration on the meat would drive a proportion of samples below the detection limit and consequently the prevalence would be considered to be lower.

Interestingly, no decreasing trend was observed in chilled broiler meat at retail. This may seem odd, since the prevalence of *Campylobacter* in broilers has been found to be a predictor for the occurrence in the meat (manuscript II). However, the strong seasonality associated with the prevalence may have significant influence. Reasons for not observing a decrease in the Danish chilled meat at retail, even though it was observed for broilers, may include an artefact of sampling. The sampling scheme for the retail surveillance is designed so that samples are collected from a variety the different products available in the display counter. This sampling includes meat from large as well as smaller producers, and may lead to an overrepresentation of meat from the smaller producers when compared to the actual sale. Meat from the smaller producers often consist of special productions, such as organic production or other types of productions with meat from older birds with a higher probability of being positive for *Campylobacter (8)*. Furthermore, the smaller slaughterhouses do not have the possibility of implementing preventive measures such as scheduling. Accordingly, *Campylobacter* occurrence based on sampling at the retail level will not reflect the exact picture of the slaughterhouse surveillance nor the broiler prevalence.

The increasing trend of *Campylobacter* occurrence on Danish frozen meat may reflect the scheduling strategy of meat from positive flocks to frozen production. Directing meat from positive flocks to freezing will result in a prevalence increase. Though, end products will be expected to

pose a lower risk to consumers, compared to a scenario where the same products was sold chilled, because of the reduction of concentration as a result of freezing.

The seasonality in *Campylobacter* prevalence observed for Danish chilled meat was to great extent associated with seasonality in broiler flock prevalence, which most probably is the causal factor of the seasonality in meat. The seasonality in meat may be influenced by additional factors, though, to a lesser extent; biological or circumstantial (manuscript II). For example, during warm periods, there is an increased pathogen infection pressure *(51, 52)* and it is less feasible to comply with the freezing scheduling strategy due to a larger proportion of positive flocks going to slaughter.

The less distinct seasonality observed for the other categories of meat (Danish frozen meat and imported, chilled and frozen meat) may be influenced by different factors obliterating the impact of season (manuscript II). Seasonality of *Campylobacter* prevalence for chilled import was probably affected by the pooling of data for analysis from samples of meat of different country origin. The longer shelf life of frozen meat, compared to chilled meat, can result in an extended time before the products become available to the consumer as companies can store the products and release them according to demand. Furthermore, the products will be available in the counter at retail for a longer time. Consequently, it is reasonable to expect seasonality in frozen meat at retail to be less distinct than in chilled products.

The first Danish action plan, implemented to control *Campylobacter* in Denmark, has likely influenced the prevalence in broiler flocks and Danish chilled broiler meat. A small decrease in the number of human cases has also been observed after 2001-2002 (manuscript I). The explanation for the effect on human cases not being more significant is probably due to other factors counterbalancing the effect of the implemented interventions. First of all, the first initiatives and action plan only targeted the broiler meat produced in Denmark. However, a large share of broiler meat consumed in Denmark is in fact imported (approximately 40% in 2008) and the strategy would have had no effect on cases caused by this meat. The case-by-case initiative, which targets both Danish and imported meat addresses this problem, and was introduced in 2007. Furthermore, the sources of *Campylobacter* are many and the transmission routes are various. Even though broiler meat is believed to be the largest single source of human campylobacteriosis, broiler meat only accounts for a fraction of human cases. The exact fraction of human cases attributable to broiler meat is not known. Finally, the risk of acquiring campylobacteriosis is also influenced by numbers of *Campylobacter* on the meat.

In the new four year action plan, which was initiated in 2008, risk managers have focused, among other initiatives, on the development of fly screens for broiler houses, which seems to be the most promising method to efficiently reduce the prevalence of *Campylobacter* in broilers in Denmark. Studies have indicated that by adding fly screens to broiler houses, the summer peak potentially can be eliminated *(52)*. The potential of this work seems incredibly important when related to the marked seasonality of *Campylobacter* in Danish chilled broiler meat which is strongly influenced by broiler prevalence *(22)*; potentially reducing the human risk from the Danish produced broiler meat. The implementation of fly screens could potentially result in a

Campylobacter prevalence baseline of 10 to 20% of broiler flocks all year around *(98)*. A scenario of obtaining a prevalence level of 10% all year around has been estimated to result in a human risk reduction of 48% compared to the present situation *(33)*.

Campylobacter prevalence is high in cattle, pigs, and broilers but a high occurrence on meat is only found for broiler meat. The main factor contributing to this is believed to be the way the animals are handled during slaughter; including less faecal contamination and more extensive dying-off of *Campylobacter* on drier surfaces in the pig and cattle processing compared to processing of broiler carcasses. Cattle and pig carcasses are split during processing as opposed to broiler carcasses that are handled whole, which complicates the cleaning of the birds after evisceration. Also broilers are slaughtered at high speed and using large amounts of water, both factors that can contribute larger amounts of faecal contamination on broiler carcasses, compared to carcasses of pigs and cattle. It has been shown that the feather removal and evisceration process are of most impact in relation to contamination during slaughter *(105)*. QMRA's have emphasized the importance of reducing the numbers of *Campylobacter* on broiler meat in relation to effective human risk reduction *(86)*.

6. INTERVENTIONS AT SLAUGHTER (manuscript III)

Different strategies may be applied to reduce the number of *Campylobacter* in broiler meat. As part of the Danish strategy, strict biosecurity measures have been implemented in the Danish primary production of broiler chickens to bring down the *Campylobacter* prevalence, while at the slaughterhouses the strategy aims to reduce the concentration of *Campylobacter* on contaminated meat by applying physical decontamination measures. This chapter only concerns interventions at slaughter, because interventions in the primary production were not within the scope of this thesis.

At slaughter, a reduction in the concentration of *Campylobacter* may be accomplished by either preventing *Campylobacter* contamination of the carcasses during the slaughter process, or by reducing the concentration of *Campylobacter* on the carcasses through decontamination procedures. Decontamination should always be considered a supplement to and not a substitute for good hygiene practices (GHP). However, under the current processing conditions, GHP cannot completely prevent *Campylobacter* contamination from intestinal contents to the surface of broiler carcasses. Carcasses are primarily contaminated during the processes of feather removal and evisceration *(105)*, and since broilers are often colonized by *Campylobacter*, implementation of control measures at this stage of the production is essential, to reduce the risk of human infection.

The optimal control measure is cheap and effective without causing any changes to the characteristics of the fresh meat, and is acceptable to the consumers. Multiple studies have been published on different physical decontamination techniques; however, no technique has been demonstrated to fulfil all the characteristics of an optimal control measure, as outlined above. The effect of chemical decontamination has also been studied but cannot be implemented since, at present, no chemical substances have been approved for decontamination of foods of animal origin in the EU *(33)*. Furthermore, no sound solution for handling the hygienic processing challenges in the broiler production has been found yet.

In Denmark, part of the action plan to control *Campylobacter* at the slaughterhouse, has been to freeze meat from positive flocks, to the extent possible (meaning to the extent practicable as a consequence of limitations of the methods used for detecting *Campylobacter* and with regard to consumer demands for special products that require the meat to be fresh, e.g. chilled meat). Furthermore, forced air chilling of carcasses and crust freezing, of breast fillets only, has been implemented at the major slaughterhouses. In theory, these techniques may have a *Campylobacter* reducing potential due to the effect of drying out the surface of the meat and freezing of the surface, respectively.

A decontamination technique combining steam and ultrasound to reduce surface contaminating microorganisms, as for example *Campylobacter*, has been under development in Denmark (by FORCE Technology). The technology is based on the *Campylobacter* reducing effect of hot steam. This effect is enhanced by application of ultrasound, which sets the protective layer of air and vapour around the broiler carcass in motion, allowing the steam to reach the bacteria within microstructures and cavities of the surface more easily.

In the following, the reduction potential of forced air chilling, crust freezing, and steam-ultrasound were investigated and compared to the effect of freezing. The additional contamination on carcasses caused by visceral rupture during evisceration was also examined. Finally, effects of decontamination on varying levels of *Campylobacter* contamination were assessed to explore the influence of specific strains and of the initial contamination level on *Campylobacter* reductions.

6.1 Materials and Methods

6.1.1 Interventions at slaughter - Study design

Investigations of the reducing potential of the three different physical decontamination techniques and the level of contamination caused by visceral rupture were performed at a major Danish slaughterhouse using carcasses from naturally *Campylobacter* colonized broiler flocks (as determined approximately one week prior to slaughter).

The effect of decontamination techniques were assessed based on enumeration of *Campylobacter* in whole carcass wash from carcasses before (controls) and after treatment. In total, 50 samples were collected from each of three flocks (n=25 before and n=25 after treatment) for forced air chilling, crust freezing, and visceral rupture. Furthermore, 60 samples per flock were collected from each of two flocks for evaluation of steam-ultrasound. The number of samples was based on sample size/power calculation.

Results were compared to the effect of freezing from an evaluation at the same plant in a previous study *(105)*.

A detailed description of the methods and analyses is provided in manuscript III.

6.1.2 Decontamination effect at different levels of contamination (manuscript IV)

Examination of the influence of contamination level and strain on the magnitude of reduction of physical (freezing for 24 hours and 7 days) and chemical (tartaric acid and tri-sodium phosphate) decontamination measures were carried out in the laboratory with artificially contaminated samples.

An assessment of the effects was based on surface rinsing of control and treated samples. Studies of each measure included four levels of contamination and three strains and the studies were performed in triplicate.

A detailed description of the methods and analyses is provided in manuscript IV.

6.2 Results and Discussion

The evaluation of decontamination measures implemented in the major Danish slaughterhouses (forced air chilling, crust freezing and freezing) with the potential to reduce numbers of *Campylobacter* all showed effective to greater or lesser extent. (Detailed description of results is presented in manuscript III).

Of the already implemented techniques, freezing proved to be the most efficient (reduction of 1.4 log cfu/sample). This was the immediate reduction, the number of *Campylobacter* is expected to decrease further during prolonged storage, however, at a slower rate than during the immediate freezing. Equal results have previously been demonstrated by others *(47, 107)*. The reduction obtained by forced air chilling and crust freezing, were similar, approximately 0.4 CFU/sample for both techniques. Even though crust freezing is a short lasting freezing of the surface of the meat, where *Campylobacter* contamination is primarily found, the reduction obtained did not match the effect of freezing. An explanation for this may be that the rapid freezing, using a lower temperature gradient, causes smaller ice crystals and thus less damage to the bacterial cells than ordinary freezing.

Ordinary freezing changes product characteristics, from chilled to frozen meat, why this control measure cannot be used uncritically. The option of freezing all domestically produced, contaminated meat is not feasible for the Danish market, as this would cause a shortage of chilled products during periods, where *Campylobacter* prevalence in broilers is high. This may lead to increased import of chilled broiler meat in order to sufficiently meet the market demands. Since imported, chilled meat may also be contaminated, this would not solve the *Campylobacter* problem. Furthermore, since only a fraction of broiler flocks positive at slaughter is identified prior to slaughter *(33)*, it is of the essence to identify other potential methods to reduce the *Campylobacter* concentration.

The mean reduction obtained with the steam-ultrasound treatment was large (≥2.5 log cfu/sample), however reductions obtained between flocks varied greatly. Furthermore, the treatment induced an adverse effect of a boiled appearance of carcasses. Since these studies were carried out, considerable work has gone into the optimization of this technique with regard to obtaining a consistent effect and acceptable appearance of treated products. Current results from adjusted equipment have not been able to accomplish the same reduction as seen in the initial experiments, but the appearance of products has been considerably improved as a consequence of reduced treatment. The technique has shown promising results, obtaining reductions around one log cfu/g (analyses of skin obtained from treated carcasses)[11], but more studies are needed with the on-line equipment to demonstrate the actual effect in an industrial scale processing *(101)*. Significant *Campylobacter* reduction and maintenance of product quality has been difficult to achieve with steam treatment alone because of a boiled appearance of the skin or meat surface *(69, 121)*.

Chemical treatments are generally reported to reduce *Campylobacter* levels in a magnitude of 0.5 to 2 log units *(76)*. Effects of chemical treatment cannot always be separated from the control treatment using water alone. Thus, the effect of the chemical substance versus the effect of treatment using only water (washing off the bacteria) is not always reported *(12, 33)*. In spite of inconsistencies in the ways the results are reported, there are no indications of the existence of a single substance, meeting all the characteristics of an optimal control measure, awaiting approval.

[11] Evaluation is based on results from studies on the slaughter line with natural *Campylobacter* contaminated broilers.

No single decontamination technique, applicable within legislation, is able to eradicate *Campylobacter* completely, or even reduce the number of *Campylobacter* to negligible levels, from contaminated meat, without changing product characteristics. To improve the *Campylobacter* reduction, a 'multiple hurdle' approach may be used, applying several control measures in sequence obtaining a combined or synergetic decontamination effect. The efficacy of such an approach has not been documented with regard to *Campylobacter* in the broiler production. However, the approach of multiple-sequential interventions for decontamination purposes have been shown to be an effective method for reducing microbiological contamination of beef carcasses *(16)*.

In addition to efforts to reduce *Campylobacter* contamination on meat, without influencing the product, an effective measure against *Campylobacter* is cooking. Heat treatment has been estimated to obtain a 100% human risk reduction (given no re-contamination). This measure is, however, not relevant when focussing on the consumer demand for fresh meat.

The EU baseline survey on the prevalence of *Campylobacter* in broiler batches and of *Campylobacter* and *Salmonella* on broiler carcasses, in 2008 *(40)* found that the likelihood of carcasses being contaminated with *Campylobacter* and the likelihood of carcasses having high *Campylobacter* counts varied significantly between countries and between slaughter-houses within countries. These findings indicate that certain slaughterhouses are more capable than others in preventing *Campylobacter* contamination and in controlling the contamination and/or the *Campylobacter* counts on the carcasses *(40)*. Assuming that different steps in slaughter processing will influence the *Campylobacter* occurrence in equal manner (scalding, feather removal, evisceration, etc.) *(105)*, other risk factors may influence the differences observed between slaughterhouses. A study by Habib *et al*. *(50)* used the survey data from the EU baseline study combined with a national scoring system for general slaughterhouse quality management, including evaluation of the operational hygiene to assess factors associated with *Campylobacter* contamination in Belgian slaughter houses. The authors found that deficiencies in slaughterhouse quality management and operational hygiene were correlated with *Campylobacter* contamination on broiler carcasses. The slaughterhouse effect is likely related to several factors; however, the findings indicate that hygienic operations contribute to *Campylobacter* contamination. Optimizing the slaughter hygiene will most likely influence the *Campylobacter* occurrence on broiler carcasses (prevalence as well as concentration). However, methods or equipment for improvement of slaughter hygiene in relation to limiting the contamination from faecal leakage and cross-contamination are rarely described.

A proportion of carcasses are contaminated during slaughter following visceral rupture (the target in most Danish plants is a rupture frequency of less than 5%). In own studies, visceral rupture was assessed to cause an additional contamination of approximately one log unit for carcasses with visual faecal contamination. These findings are in accordance with those of Berrang *et al*. *(18)*, who found increasing *Campylobacter* contamination on carcasses with increasing amounts of faecal material. It is likely that hygienic measures could reduce this contamination, for example by optimizing the hygienic design of equipment to physically prevent or remove faecal contamination (e.g., by extra washing of carcasses).

Changes to the standard broiler processing steps have been addressed to reduce *Campylobacter* contamination at the slaughterhouse *(17, 84)*. Different means of cloacal plugging before defeathering have been tested. Cloacal plugging before culling resulted in significantly lower *Campylobacter* concentration and prevalence on plugged carcasses after feather removal compared to controls *(84)*. Variation over the cloacal plug has been examined, but was not found to be effective *(17)*. The applicability of the plugging technique is limited, as the work would be labour-intensive and not especially practical. A practical method to markedly prevent or reduce faecal contamination during processing has not yet been developed. However, Berrang *at al*. *(17)* investigated changes to the order of standard broiler processing steps with regard to the evisceration process and found that performing evisceration prior to scalding was effective in significantly moderating the increase in *Campylobacter* on broiler carcasses during automated feather removal. Accordingly, changing the order of standard broiler processing may help to control contamination with *Campylobacter*.

Action plans against *Campylobacter* have been implemented in several countries with productions similar to the Danish production. The focus is primarily on a high level of biosecurity in the primary production. Interventions at slaughter is more rarely described *(37)*. Though, freezing is described as control measure in the production of meat from positive flocks in the Icelandic and Norwegian control strategies *(66, 117)*. In the Norwegian strategy, heat treatment is described as an alternative to freezing *(66)*. Other mitigating measures at slaughter have not been described.

The human health impact of available decontamination techniques has been assessed in relation to human risk reduction *(33, 59)*. The approaches used for these purposes involve the use of risk assessment modelling. Human risk reduction of 90-100% has been estimated for the application of freezing, cooking and irradiation as intervention measures against *Campylobacter* (assuming that products are not re-contaminated). Furthermore, the use of steam, hot water and chemical decontamination (e.g. TSP) has been estimated to obtain considerable human risk reduction.

The effect of a given control measure will depend on a number of factors; including production conditions, and where in the production line the measure is applied. Production conditions like temperature and humidity or equipment design may influence effects of interventions in different ways, e.g. temperature of carcasses or a chemical substance, or hot versus cold water may influence the efficacy of the treatment *(97, 118)*. Where in the production chain a control measure is inserted may affect the efficacy directly or by the potential risk of re-contamination. For example, the effect of chemical decontaminants may be different if applied in the scalding tank compared to direct application e.g. by spray before chilling *(59)*.

Interventions can be applied to either all broiler flocks or by scheduling of positive flocks for treatment *(33)*. The first option is more expensive as more birds need to be treated, however, due to existing methods and logistics, treatment of all positive flocks cannot be secured with scheduling. This renders a prioritisation of economy versus effect.

Evaluation of interventions in relation to human risk reduction using QMRA requires representative data to infer realistic estimates. The Opinion on control options in relation to

Campylobacter in the broiler meat production developed by the EFSA concludes: "Another general finding is that it is difficult to obtain good representative data that allow estimating the effect of specific control options in terms of reduction in *Campylobacter* concentration or prevalence. Quite often the effect estimates are based on one or a few published or unpublished laboratory experiments, or expert opinion, and they cannot always be correctly applied to conditions other than the specific ones under which they were designed. As a consequence their predicted effects on risk reduction are also highly uncertain" *(33)*.

The strength of decontamination study data have been defined, based on design, in relation to the use of artificial inoculated or naturally contaminated samples tested in laboratory, pilot or industrial scale *(33)*. In relation to the general body of evidence for interventions, studies using naturally contaminated samples in industrial scale were ranked the highest, while studies with artificial inoculated samples in laboratory scale were ranked the lowest.

Multiple studies on reduction of *Campylobacter* on broiler meat by decontamination (physical or chemical) at slaughter using artificial inoculation have been published; but, the influence of different inoculation levels on reductions and strain specific effects are rarely included in the investigations. Studies have often used only high inoculation levels (10^7-10^8 cfu) and/or one single strain for evaluation. This issue, however, is of great importance, if the magnitude of reductions is influenced by the initial concentration, and may add to the uncertainty in predicting the effects on risk reduction.

We found that log reductions tended to be unaffected by the initial *Campylobacter* concentration on the meat for the decontamination techniques investigated. Though, reductions obtained by freezing (at -20°C for seven days) and treatment with TSP (marginally significant) were exceptions to this. Analyses suggested that different reductions were obtained using high inoculation levels (10^7 cfu/sample) compared to the lower levels 10^3-10^5 cfu/sample. Furthermore, reductions obtained by freezing, treatment with tartaric acid and treatment with TSP varied significantly for the strains tested. Significant strain variation in treatment of broiler meat with organic acids have also been reported in other studies *(21, 49)*. Accordingly, reductions obtained in studies with one or few *C. jejuni* strains may not represent the general result for the species.

These results support the recommendation from EFSA *(32)* and the criteria proposed by FAO/WHO *(44)*, declaring that the highest body of evidence of data, which are to be used in efficacy evaluations and risk assessments, should preferably be from industrial scale studies using naturally contaminated meat. If investigations of naturally contaminated meat cannot replace inoculation studies, a mixture of strains found in the production environment, at levels as close to the natural contamination as possible should be used.

Different strategies may be chosen with regard to interventions against *Campylobacter* at the slaughterhouse; chemical, physical and/or hygienic measures. At present, no chemical substances have been approved for decontamination of broiler meat within the EU. The development of hygienic design of processing equipment is limited (or has not been described). Therefore, in Denmark, the primary focus has been on physical decontamination measures where freezing has

shown most potential. Another Danish study has investigated the *Campylobacter* reducing potential of marinating ingredients *(21)*, and the potential was considerable. However, marinating meat can only be applied to a fraction of the production, as this type of product is presently not in great demand by consumers in Denmark.

Hygienic measures at slaughterhouses may be optimized, but as long as contamination of carcasses cannot be avoided, the application of concentration reducing measures is a sensible approach in relation to decreasing human risk of infection from broiler products, because concentration is believed to be a significant factor in relation to the probability of inducing human illness *(86)*.

Individual control measures, applicable within legislation, have been unable to reduce *Campylobacter* contamination to negligible levels without changing product characteristics. Further studies and development of hygienic and decontamination measures are therefore needed. A relevant aim of future studies could be to develop a cheap and effective measure that does not change the product characteristics of fresh meat and which will be accepted by consumers. As this has been a focus area for some time, extensive research has already been carried out, and perhaps a change in strategy may be needed; for example more emphasis should perhaps be put on the development of ‘multiple hurdle’ strategies.

7. EVALUATION OF THE IMPLEMENTED ACTION PLANS (manuscript V) - Temporal assessment of human risk of *Campylobacter* from retail broiler meat, from 2001 to 2010

In Denmark, several interventions have been implemented as part of the Danish action plans against *Campylobacter* in broilers and broiler meat, from the primary production to the consumer level *(102)*. The Danish strategy to control *Campylobacter* coincided with a reduction in the prevalence in broiler flocks and broiler meat. A small decrease in the number of human cases after 2001-2002 has also been observed. The explanation for the decrease in the number of human cases not being more noticeable is probably due to other factors counterbalancing the effect of the implemented interventions (manuscript I).

The Danish strategy to control *Campylobacter* focuses on the production of broilers and broiler meat. Broiler chicken has been recognized as the most important single source of human campylobacteriosis; however, human cases are also attributed to other sources and the exact proportion attributable to broiler chicken is not known. This may influence the assessment of implemented measures, if based on the decrease of human cases alone. Furthermore, an evaluation performed on register data cannot be compared directly to the situation where no measures have been implemented, and one should be careful inferring direct causal relationship as a number of factors may influence the *Campylobacter* status in both human and broilers.

As opposed to evaluating the Danish *Campylobacter* situation and the effect of the action plans based on changes in the registered number of human cases, the *Campylobacter* status has traditionally been assessed based on the evaluation of prevalence in for example broiler flocks and broiler meat. For *Campylobacter*, the number of bacteria ingested is believed to be of great importance in relation to human illness *(86)*. Hence, an assessment of the implemented measures, in relation to impact on human risk reduction, would probably be more accurately portrayed by inclusion of data concerning *Campylobacter* concentration. Accordingly, the use of quantitative risk assessment models could be another way of evaluating changes in human risk.

7.1 Quantitative microbiological risk assessment

Quantitative microbiological risk assessment is a science based discipline combining empirical data, theory and hypotheses in mathematical models to obtain a simplified representation of reality. Models are setup to assess specific questions or scenarios. QMRA modelling regarding *Campylobacter* is commonly used to assess the human risk due to *Campylobacter* in broiler meat, and to evaluate potential effects of control measures in the broiler production chain *(86)*. In Denmark, risk assessment models are also used on a day to day basis in the case-by-case control of e.g. *Campylobacter* in fresh meat.

Risk assessment is one component of a three element risk analysis paradigm encompassing risk assessment, risk management and risk communication. Risk analysis has emerged to improve food control systems, and is a way to consider the entire production of a specific food, in a chain perspective. Quantitative risk assessments are important tools for supporting risk management

decisions towards a specified risk; comprising hazard identification, hazard characterization, exposure assessment, and risk characterization. Risk assessment should be performed based on a specific question, formulated by risk managers, providing an objective evaluation of the given problem. The risk management element concerns the evaluation of risk; using the information gathered during risk assessment integrated with social, cultural, economic, and political considerations; taking into account the control measures available and appropriate actions to reduce unacceptable risk. Risk communication concerns communication of information between risk assessors, risk managers, and stakeholders (e.g. producers, the general public and specific subpopulations subject to increased risk). It is of the utmost importance that both risk assessors and managers agree on the formulation of the specific issue of concern, in order to obtain a mutual perception of the question to be handled. Thus, an initial and thorough problem formulation is of major importance. All elements should be transparent and independent, but interactive.

Several countries have developed QMRA's for *Campylobacter* in broilers and broiler meat; e.g. the European countries Great Britain *(58)*, Denmark *(104)* , the Netherlands *(87)*, Germany *(23)*, and also a more general assessment was developed in the auspices of FAO/WHO *(45)*.

In the construction of models, in a food chain perspective, different approaches as well as different levels of complexity may be used. Different models incorporate different stages of the production chain based on the question or scenario that the model is set up to assess. The Danish, Dutch, and German models illustrate such differences in approaches well. For example, the Dutch model covered the entire production chain from the primary production to human illness, in order to advise risk managers in the assessment of multiple interventions throughout the food chain, as well as in comparison of the effects of different interventions *(87)*. The Danish model considered processing at the slaughterhouse in relation to human illness with the aim of providing Danish risk managers with information on the relative importance of different interventions on the number of human cases associated with *Campylobacter* in Danish retail chicken *(104)*. In contrast, the German model comprised only a minor fraction of the chain, from retail level to human illness to assess how chicken prepared at home exposes the consumer to *Campylobacter (23)*.

The QMRA models are sensitive to the assumptions and hypotheses incorporated. Especially the consumer phase module is shrouded with unknown factors, for which several assumptions need to be made. Additionally, consumer behaviour is likely to be subject to great variation regarding e.g. consumer handling of the product, bacterial transfer rates during preparation, and to which extent cross-contamination occurs, etc. Furthermore, the empirical data for dose-response models are sparse. However, using outputs to estimate the relative impact of different scenarios reduces the importance of the ambiguity. Relative risk estimates have a smaller level of uncertainty than absolute risk estimates due to the fact that similar uncertainties will be canceled out if absolute risk estimates are divided *(85)*.

7.2 Materials and methods

In the following, QMRA modelling was used as a tool to evaluate changes over time in the relative risk of human campylobacteriosis from broiler meat. The purpose was to assess the *Campylobacter* status of broiler meat at retail in the period from 2001 to 2010, coincident with the implementation of different control measures as part of the Danish action plans which were initiated in the same period.

7.2.1 Data and setup

The data used in the assessment, prevalences and concentrations, were derived from the Danish *Campylobacter* surveillance of broiler meat at retail from 2001 to 2010; categorized as domestically produced or imported meat and chilled or frozen. Samples were randomly collected nationwide from local retail establishments or whole sale. The data were considered to be representative for all retail meat available for consumption. Microbiological analysis of samples provided semi-quantitative estimates of concentration.

The QMRA model does not differentiate between whole broilers and parts of fresh products; accordingly assuming that the risk, given a certain concentration, is independent of the type of product. This is probably not the factual truth due to the different attributes of the cuts (e.g. skin/no skin) as well as different processing steps, which could be of influence, but the assumption was necessary as the model does not account for different cuts.

Data on the amount of broiler meat available for sale in Denmark was obtained from the Danish Agriculture and Food Council and Statistics Denmark.

Microbiological analysis of collected samples and preparation of data for model input is described in manuscript V.

Estimates of prevalence, mean concentration, and standard deviation of the concentration, for each meat category for each year, were obtained using a maximum-likelihood-estimation (MLE) method. This method was chosen for two reasons. Firstly, some kind of approximation was needed to compute distribution for application in the QMRA model. Secondly, because the available data is only a sample of all the meat available for sale and we would like to be able to conclude on the whole population (all meat available for sale). Accordingly, data was used as a representative base for fitting a distribution to infer the variation for the whole population.

Estimation of the relative risk in total from broiler meat available for sale in Denmark was computed from the observed data from the four meat categories. Evaluation of the relative risk in total from broiler meat available for sale in Denmark could not be done directly from the MLE fitted data as the model was not converging for some of the data. This approach was considered to be a useful alternative as relative risk estimates from using either the MLE fitted data or the observed data were similar.

7.2.2 Risk model

The QMRA model used to evaluate the *Campylobacter* situation was described by Nauta *et al*. *(88)*. The model was applied as is, using the estimated prevalence, mean concentration and standard deviation of the concentration as input. The output of the risk assessment model is the mean probability of illness from consumption of a random sample of broiler meat.

Relative estimates of risk were compared on a yearly basis from 2001 to 2010, considering Danish chilled meat in 2007 as the baseline with relative risk 1. The year 2007 was chosen as baseline for the evaluation as this was the year before the implementation of the second Danish action plan (2008-2012)

7.3 Results and Discussion

7.3.1 Data

The data used for the assessment was semi-quantitative. A maximum likelihood approach for censored data can be a way to prepare the available data for application in the QMRA. The semi-quantitative surveillance data were approximated to lognormal distributions by maximum likelihood estimation, producing estimates of prevalence, mean concentration and standard deviation of the concentration. The accuracy of the fit will vary depending on the data.

The performance of the MLE is illustrated in Appendix figure 1. The estimated prevalence was generally higher compared to observed prevalence. This was the expected outcome. With the MLE, a proportion of samples below the detection limit will be considered as positives and consequently the computed prevalence will be higher than the observed. Estimated prevalence and observed prevalence were approximately related linearly. The correlation between the estimated mean concentration and the observed mean concentration was linearly related for high mean concentrations, indicating satisfactory estimation at high concentrations. This is plausible, as high mean concentrations in most cases will be derived from data comprising a larger proportion of positive samples; constituting a larger data basis for computation. At lower mean concentrations, the association between observed and estimated concentration was more dispersed; probably due to the lower number of positive samples to infer distributions from. The MLE method computes lower mean estimates of the concentration, as a proportion of samples below the detection limit will be considered positive.

7.3.2 Application of QMRA

The *Campylobacter* intervention strategies have been implemented with the aim of decreasing human risk of campylobacteriosis from broiler meat. Traditionally, the *Campylobacter* status is evaluated based on prevalence estimates; however, the observed prevalence was not a very good surrogate measure for human risk (Appendix figure 2). The observed mean concentration appeared to be more closely related to the human risk than prevalence, but still, did not seem to be a good sole predicting measure (Appendix figure 2). The application of QMRA as a tool to assess the effect

of intervention strategies proved to be more relevant. Using QMRA has an added value compared to an evaluation of the results in terms of prevalence or mean concentrations alone. The approach of combining prevalence, mean concentration and also the variability of the mean concentration is important in relation to the probability of inducing human illness. As concluded by Nauta *et al*. *(86)*, the tails of the distributions describing the variability in *Campylobacter* concentrations between meat products and meals determine the risks, not the mean values of those distributions.

The use of QMRA is a simplified way of portraying real life scenarios. This approach adopts several assumptions and hypotheses, which each in their own way will influence the outcome of the modelling. The modelling provides a mean estimate of human risk from a random sample of broiler meat. Consequently, this estimate concerns only conditions comprised in the model; not including people eating raw chicken sushi, etc., and furthermore, for some groups the risk would be higher because of biological reasons; e.g. young children, elderly people, and immune-compromised persons.

We used a static model to infer changes over time; disregarding any temporal changes other than prevalence and concentration; consequently, disregarding any other conditions subject to change over time. One of the intervention measures in the action plan was to conduct consumer campaigns to reduce the cross-contamination in domestic kitchens. These have not been considered in the evaluation of the action plans as it is not feasible to measure potential changes, however, the impact is not assumed to be of importance, as studies has demonstrated the difficulty in actually changing habitual behaviour of consumers *(89)*.

7.3.3 Risk from chilled and frozen meat

Evaluation of the temporal changes in human risk from broiler meat available for sale in Denmark in the period 2001-2010, inferred from QMRA based on surveillance data is presented in manuscript V. The detailed fluctuations in human risk from the four meat categories are presented in this manuscript. In brief, human risk was generally higher from chilled meat compared to frozen. The most evident changes were the decreasing risk from chilled imported meat in the latter part of the studied period (2005-2010) and the increasing risk from Danish frozen meat (2003-2010).

The first Danish *Campylobacter* action plan was launched in 2003 and targeted the domestic broiler production (described in section 5.2.1). The increasing risk from Danish produced, frozen broiler meat coincided with the control measure of scheduling of meat from *Campylobacter* positive broiler flocks to frozen production to the extent possible. The allocation of meat from positive flocks results in a frozen production with a higher *Campylobacter* prevalence. Accordingly, the produced meat may still be positive but of lower risk, as the freezing reduces the *Campylobacter* numbers on the meat. The increase in risk from frozen products was not associated with a direct reduction in risk from chilled meat, which could have been expected. This may be influenced by increasing concentration in chilled meat, which may maintain the risk and by the fact that the increasing human risk from Danish frozen meat was probably caused by the scheduling of meat which is carried out at the large slaughterhouses, whereas the chilled production is influenced by an

increasing production of special products which are bound by retail orders and therefore are not included in the scheduling effort; e.g. meat from broilers reared on special feed (maize, flaxseed, etc.), neutral marinated meat, among others. The result is probably further influenced by samples from smaller slaughterhouses that are not able to implement control measures as scheduling.

The effect of scheduling *Campylobacter* positive broiler flocks to the production of frozen meat may be increased if the identification of positive flocks could be improved; i.e. if the *Campylobacter* status of flocks could be determined closer to the time of slaughter. At present, the evaluation of flock status is around one week prior to slaughter, identifying approximately 50 % of flocks being positive at the time of slaughter *(65)*. A later identification may improve the performance of the intervention measure. However, the logistics may be more difficult, because the response time from obtaining knowledge of flock status to slaughter will be shorter and the fact that the flexibility in production cannot be determined by flock status alone, but are influenced by retail orders at the slaughterhouse, obligating the production of specific proportions of chilled and frozen meat etc. Especially in the summer period, the higher *Campylobacter* prevalence in broilers may complicate the allocation of meat.

The reduction of human risk from imported chilled meat coincides to great extent with the implementation of case-by-case evaluations. The case-by-case risk assessments have provided the retailers with an incentive to heighten standards for their suppliers. How retailers managed this task cannot be described, as the details are kept within the companies.

Half way through the second action plan, additional risk reduction was not observed. Several initiatives are still not fully processed, so the effects cannot be determined; e.g. investigation of possible implementation of hygienic measures, fly screens, etc. According to preliminary studies, the potential of installing fly screens on broiler houses have a huge potential in relation to reducing prevalence in broilers in the summer *(52)*.

No seasonality was evident for the amount of broiler meat available for consumption[12] (data not shown). Therefore, it is assumed that seasonality is not influenced by changing patterns in broiler meat consumption; e.g. consumption of frozen meat in winter and chilled meat in the summer.

The relative risk in total from broiler meat available for sale in Denmark is presented in Figure 7. Changes in risk can be observed within categories, though the total level of relative risk was not markedly lower in the last part of the study period (2008-2010) when compared to the period before the implementation of action plans (2001 and 2002). This coincided with the fact that human cases was not steadily decreasing, but fluctuating around a steady level in this period. An increase in risk was observed from 2001 to 2005, after which this tendency was turned around. The peak in 2005 was due to remarkably high mean concentrations for chilled meat, which was not readily explained. No coinciding increase was observed in the registered number of human cases in

[12] Under the assumption that Danish produced and imported meat is available for sale in equal ratio all year around (monthly data only available for imported meat).

the same period. It would be attractive to perform an uncertainty analysis for the relative risk estimates to evaluate the importance of the fluctuations; however, at present no method is available to perform this kind of analysis.

A validation of the retail data performed by comparing the relative human risk from Danish chilled retail meat to the relative risk estimated from surveillance data from the large slaughterhouses (accounting for approximately 98% of the Danish chilled meat) revealed a similar high estimate of human risk in 2005. In general, estimates from the retail data seem to reflect the slaughterhouse data to some extent; though in 2004, there seems to be some inconsistency that cannot readily be explained (Figure 8). Slaughterhouse surveillance was established in 2004, why data are not available for previous years.

The evaluation of the relative risk of broiler meat is dependent on the prevalence and concentration on the meat. Though, other factors may influence the risk of illness; e.g. the different types of *Campylobacter* present, which may be more or less virulent. At present, these factors have not been included in the evaluation.

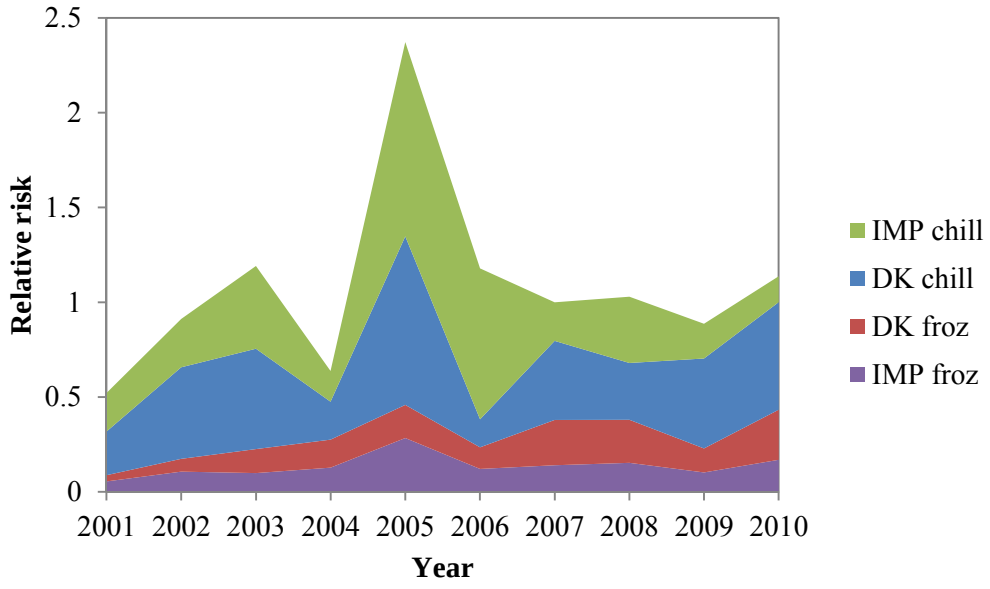

Figure 7. Total risk based on calculations using raw data for the four meat categories. Risk is calculated in relative to the total risk in 2007.

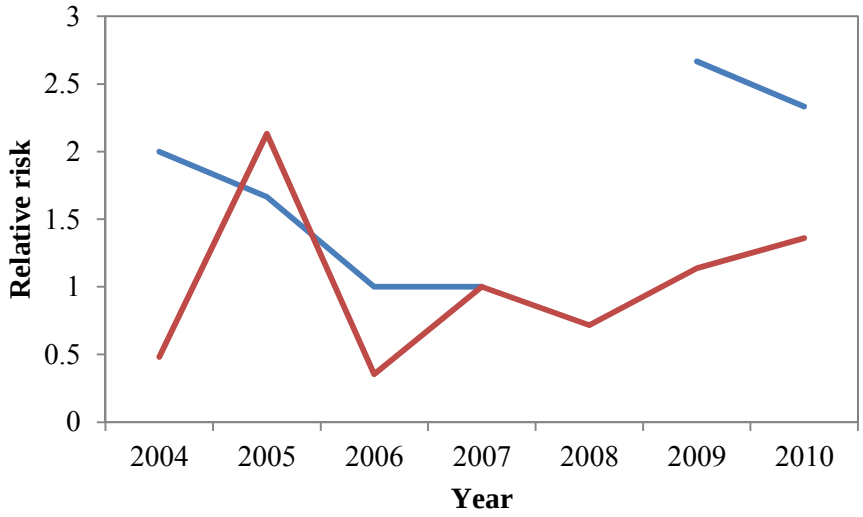

Figure 8. Comparison of relative risk estimates from Danish chilled meat based on retail data (red) and slaughterhouse data (blue), 2004-2010.

8. THE DANISH CAMPYLOBACTER SOURCE ATTRIBUTION (manuscript VI) - Source attribution of human campylobacteriosis cases caused by *Campylobacter jejuni*

Poultry, and especially broilers, has been recognized as a major reservoir for *Campylobacter* spp. and broiler meat has been established as an important vehicle for human exposure to this pathogen *(41)*. *Campylobacter* spp. are found widespread and consumer behaviour with regard to consumption of food and water, leisure activities, occupation etc. may influence the importance of different sources. Food consumption patterns vary in different countries and regions. Traditions such as consuming untreated water, raw milk and products thereof have been shown to increase the risk of human campylobacteriosis *(38, 70, 92)*. Consequently, the attribution of human campylobacteriosis cases to different sources may vary between countries and regions.

Human campylobacteriosis cases are mainly sporadic. However, *Campylobacter* outbreaks may be used to identify unrecognized sources and exposure routes that would not have been considered in regular surveillance. For example, *Campylobacter* outbreaks have been associated with swimming in natural waters *(108)*, mountain biking *(116)*, and consumption of peas (contaminated by wild birds) *(46)*. Though, sources most commonly reported as associated with *Campylobacter* outbreaks (in the EU in 2005-2006) were meat (undefined), chicken meat, and dairy products *(96)*. The sources associated with *Campylobacter* outbreaks is not strictly equivalent to the sources associated with sporadic cases. For example, raw milk and water is much more commonly associated with outbreaks, while poultry products and animal contact are more frequently associated with sporadic cases *(41)*.

The majority of human *Campylobacter* cases are believed to be sporadic and caused by *C. jejuni*. To elucidate the importance of different sources in relation to the sporadic cases source attribution may be performed.

8.1 Source attribution

Source attribution is a way to track sources or reservoirs for human illness; apportioning human cases to putative sources or reservoirs. The discipline of source attribution can be used as a tool to assist risk managers in relation to targeting control strategies and potentially help identify new areas which should be given attention. Various approaches are used for this purpose (see section 8.1.1). The choice of attribution method may depend on the given circumstances; the biology of the organism, the availability of data, and the question, which needs to be answered.

8.1.1 General source attribution methodology

The different methods of source attribution can be divided in two categories; the microbiological; comprising comparative exposure modelling and the microbial subtyping approach, and the epidemiological; comprising case-control studies and analysis of outbreak data.

The microbiological approach is based on information from isolation of the pathogen by use of microbiological methods, while the epidemiological approach is based on analysis of observational data.

A thorough description of methodology, pros and cons, are described in the Scientific Opinion on Quantification of the risk posed by broiler meat to human campylobacteriosis in the EU *(41)*.

8.1.1.1 The microbiological approach

The microbial subtyping approach is based on characterization of isolates by means of subtyping. For *C. jejuni* the Multilocus sequence typing scheme (MLST), as described by Dingle *et al. (30)*, is frequently used. The principle of this approach is to compare the subtypes of isolates from different sources with those isolated from humans. The modelling needs some degree of heterogeneous distribution of subtypes among the sources, anchoring the modelling in associations between some of the dominant subtypes and specific reservoirs. A prerequisite for obtaining credible results is a representative distribution of subtypes within sources and a distribution of subtypes similar to the true distribution *(95)*. The microbial subtyping approach infers the importance of different reservoirs in relation to human illness.

Source attribution of human cases of campylobacteriosis caused by *Campylobacter* spp., using the microbial subtyping approach, have been reported by several *(81, 111, 115, 122)* and is the most frequently used method to infer the importance of different sources in relation to human campylobacteriosis.

Comparative exposure modelling is based on determining the relative importance of the known transmission routes by estimating the human exposure to that pathogen via each route. This approach requires estimates of the prevalence and concentration of the pathogen in each of the sources of exposure *(95)*. Exposure from the different transmission routes are compared to the number of human cases and the importance of each route is determined in proportion to the size of the exposure. The comparative exposure modelling approach infers the importance of different transmission routes; providing a subdivision of reservoirs.

An example of source attribution for *Campylobacter* spp. using comparative exposure modelling is the study by Evers *et al*. from 2008 *(43)*.

8.1.1.2 The epidemiological approach

Case-control studies are based on epidemiological interview information from case-patients and a group of asymptomatic control persons (assumed uninfected) *(95)*. Case-control studies are used to identify factors that may contribute to illness by comparing case-patients and control-persons with regard to exposure to various sources (e.g. food or animal) and behavioral factors. The performance of case-control investigations is the most commonly used epidemiological approach for identifying possible exposures associated with sporadic infections. Data on *Campylobacter*

immunity is sparse. Nevertheless, indications of immunity towards homologous strains have been reported. Acquired immunity may bias the risk estimates in case-control studies; underestimating the 'true' attributable risk estimates.

An example of using case-control studies to identify risk factors for sporadic cases of campylobacteriosis are the study by Wingstrand *et al*. (2006) *(123)* and the study by Stafford *et al*. (2008). The latter study additionally demonstrated the use of the risk estimates to infer source attribution.

Analyses of outbreak data is the analyses of data gathered during outbreak investigation. Such analyses can provide estimates of the importance of different vehicles for the pathogen based on the most common vehicles associated with the outbreaks *(95)*.

The use of outbreak data to quantify the contribution of different sources in relation to human campylobacteriosis is limited for *Campylobacter*, as outbreaks with *Campylobacter* spp. are rare and often caused by other sources than sporadic cases, as concluded by a study performed at European level *(96)*.

8.2 Materials and Methods

Source attribution for *Campylobacter* is complicated. The combination of a high degree of genetic heterogeneity and types being widespread among sources complicates a clear apportioning of human cases to their respective sources. Accordingly, the subtyping method used to differentiate between different types should possess the ability to discriminate between sources, but not be discriminatory to a level with no similarity.

Epidemiological studies of *C. jejuni* (e.g. case-control studies) are generally complicated due to the genetically diversity genomes. Furthermore, outbreaks are rarely detected and analysis of outbreak data was disregarded as a tool to establish the sources and transmission routes.

Various studies of source attribution for *Campylobacter* have been reported *(81, 111, 115, 122)* and interesting results have been shown using the microbial subtyping approach; e.g. in New Zealand and the UK *(83, 122)*. For the Danish source attribution of domestically acquired human campylobacteriosis, the microbiological subtyping approach was chosen. The source attribution model for *Salmonella* in Denmark, which proved to be important in the risk management of the pathogen, was based on the microbial subtyping approach *(120)*. It would be of great importance if a similar tool could be developed for *Campylobacter* for prioritization of public health resources and source specific implementation of control measures.

8.2.1 The two models used

For the source attribution of Danish sporadic human campylobacteriosis cases caused by *C. jejuni* was used two different models; the Asymmetric Island model (AI model) developed by Wilson *et al*. *(122)* and a model modified after the Danish *Salmonella* attribution model *(53)*. The

second model will be denoted the CAMSA (CAMpylobacter Source Attribution) model in the following. Both models are based on Bayesian inference using Markov Chain Monte Carlo simulation.

A detailed description of the models is presented in Manuscript VI, the models are described in brief below.

8.2.1.1 The Asymmetric Island model

The AI model apportions domestic cases to different putative sources defined by relatedness to groups comprising isolates collected from the implicated sources. It is an evolutionary model that takes the bacterial mutation, recombination, and migration into account.

The source populations of *C. jejuni* are visualized as discrete islands (each source being an island). Within each island there is a homogeneous mixing and between islands there is migration. The migration between islands may be higher between some islands compared to others. In brief, the model estimates an 'assignment probability' for each human case to belong to each implicated source; and by inference estimating the total proportion of cases attributable to these source *(122)*.

8.2.1.2 The CAMSA model (after the Danish Salmonella model)

The principle behind the model is to compare the number of human cases caused by different subtypes with the prevalence of the subtypes isolated from the different food sources, weighted by the amount of food source consumed *(53)*. The modelling needs some degree of heterogeneous distribution of subtypes among the sources, and subtypes that occur more homogeneously may then be estimated based on the prevalence of the heterogeneously distributed types. A heterogeneous distribution of subtypes is more evident for *Salmonella* compared to *Campylobacter*. Human infection may also depend on additional factors than prevalence and consumption such as survivability of the subtypes during the food processing and/or the ability to cause disease in humans (virulence/pathogenicity), and also the ability of the food sources to act as vehicles of the pathogen. Accordingly, two parameters - a subtype dependent factor and a source specific factor - are included in the model. These parameters are of unknown value and included in the model as prior distributions.

8.2.2 Collection and characterization of isolates

C. jejuni isolates were all collected in the period 2007-2008 and originated from all over the country. Samples were collected at random. In total, 406 human isolates were collected from three regions of Denmark: Northern Jutland, Funen and Zealand. Of the human isolates, 246 were reported as domestic (i.e. acquired in Denmark), 109 were reported as travel related, and 51 were with unknown travel history. Cases were reported as related to travel, if a person had stayed a minimum of one night in any other country than Denmark, within the seven days period prior to disease onset *(35)*. All human isolates were from cases characterized as sporadic i.e. not associated with any known outbreaks.

The initial sampling scheme included random sampling of nine putative sources; broiler chicken (live broilers and meat), duck meat, and turkey meat, cattle, pig, meat of lamb, pets (cats and dogs), petting zoo goats, and surface freshwater (from rivers and lakes).

Only one *C. jejuni* isolate was obtained from the sampling of pets and it was decided not to include this isolate in the modelling. Furthermore, samples from fresh water streams, petting zoo goats, milk cattle, and meat from lamb were unfortunately lost.

Isolates from the different sources were obtained from different projects; the EU baseline study of broiler carcasses *(39)*, the national *Campylobacter* surveillance for broiler meat (at slaughter plants and at retail), and the national surveillance for antimicrobial resistance (DANMAP) (including various animal species) *(7)*. Some isolates were collected especially for this study as no surveillance was carried out on routine basis (meat of lamb, pets, petting zoo goats and water).

For differentiation of isolates, characterization was attained by MLST sequence type, *flaA* type and antimicrobial susceptibility. All typing work was carried out by Statens Serum Institut and the antimicrobial sensitivity testing was performed by the Diagnostic Engineering laboratory at The National Food Institute, Technical University of Denmark.

Details regarding the specific analyses are described in Manuscript VI.

8.2.2.1 *Characterization of isolates*

The primary characterization of isolates was performed using the MLST which is a technique for typing multiple loci (specific locations) of a gene or DNA sequence on a chromosome. The procedure characterizes isolates using the DNA sequences of internal fragments of multiple (usually seven) housekeeping genes. Housekeeping genes are highly conserved. For each housekeeping gene, the different sequences are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST). Sequences that differ at even a single nucleotide are assigned as different alleles and no weighting is given to take into account the number of nucleotide differences between alleles, as it cannot be distinguish whether differences are a result of point mutation(s) or a single recombinational exchange. For characterization of isolates for the present study, the MLST scheme described by Dingle *et al*. was used *(30)*.

MLST has several advantages for studying the *C. jejuni* epidemiology. The technique is unaffected by changes in the gene order along the chromosome, which can be altered as a result of intragenomic recombination. Furthermore, the technique has proven to be highly discriminatory and easily reproduced in different laboratories *(30)*.

FlaA-typing is DNA sequencing of the flagellin gene and has been shown to be highly discriminatory for *C. jejuni* based on the large variability in a short variable region (SVR) of the gene. Characterization of *C. jejuni* from outbreaks by means of *flaA*-type has been reported to enable appointment of isolates into epidemiological relevant groups *(79)*.

Isolates were further characterized according to antimicrobial susceptibility for a panel of seven antimicrobial agents (chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nalidixic acid, streptomycin, and tetracycline) *(7)*. The result of the testing was coded as resistant (R) or sensitive (S). An isolate was categorized as resistant if resistant to at least one of the specified agents.

Genetic differentiation is essential for the apportioning of human cases to the source groups with the AI model *(122)*. The genetic differentiation between source groups was examined by Analysis of Molecular Variance (AMOVA) *(80)*. AMOVA is a method of testing population differentiation directly from molecular data.

8.2.3 Descriptive characteristics of isolates

Based on characterization by MLST, 81% of isolates from the domestic human cases were comprised within the STs also found in sources. Approximately, one third of isolates from the included sources were not found within the human isolates, especially isolates from duck meat and turkey meat were found to a lower degree in humans, when compared to other sources.

Based on analysis of molecular variance, the genetic differentiation between source categories was statistically significant for almost all groups. No significant genetic difference was found between the group comprising the domestic human cases and the group of human cases with unknown travel history. This indicates that human cases with unknown travel history were more similar to the group of domestic cases compared to the group of travel related cases.

Descriptive characteristics regarding the number of isolates and STs of the typed isolates are presented in Appendix table 4. Further descriptive analyses regarding closer study of ST grouping are performed in the auspice of Statens Serum Institut.

8.3 Results and Discussion

The AI model and the CAMSA model produced similar results. Both models attributed the vast majority of cases to the broiler chicken reservoir while the second most important reservoir was cattle (basic models) (Figure 9). The primary difference between the models is the proportion of cases which are categorized as unknown by the CAMSA model (approximately 20%). The AI model attributed 52% (CI 37-67%) to Danish chicken, 17% (CI 3-33%) to imported chicken, and 17% (CI 7-28%) to cattle. Similarly, the CAMSA model apportioned 38% (CI 28-47%) to Danish chicken and 14% (CI 10-18%) to imported chicken, while 16% of cases (CI 7-25%) were attributed to cattle.

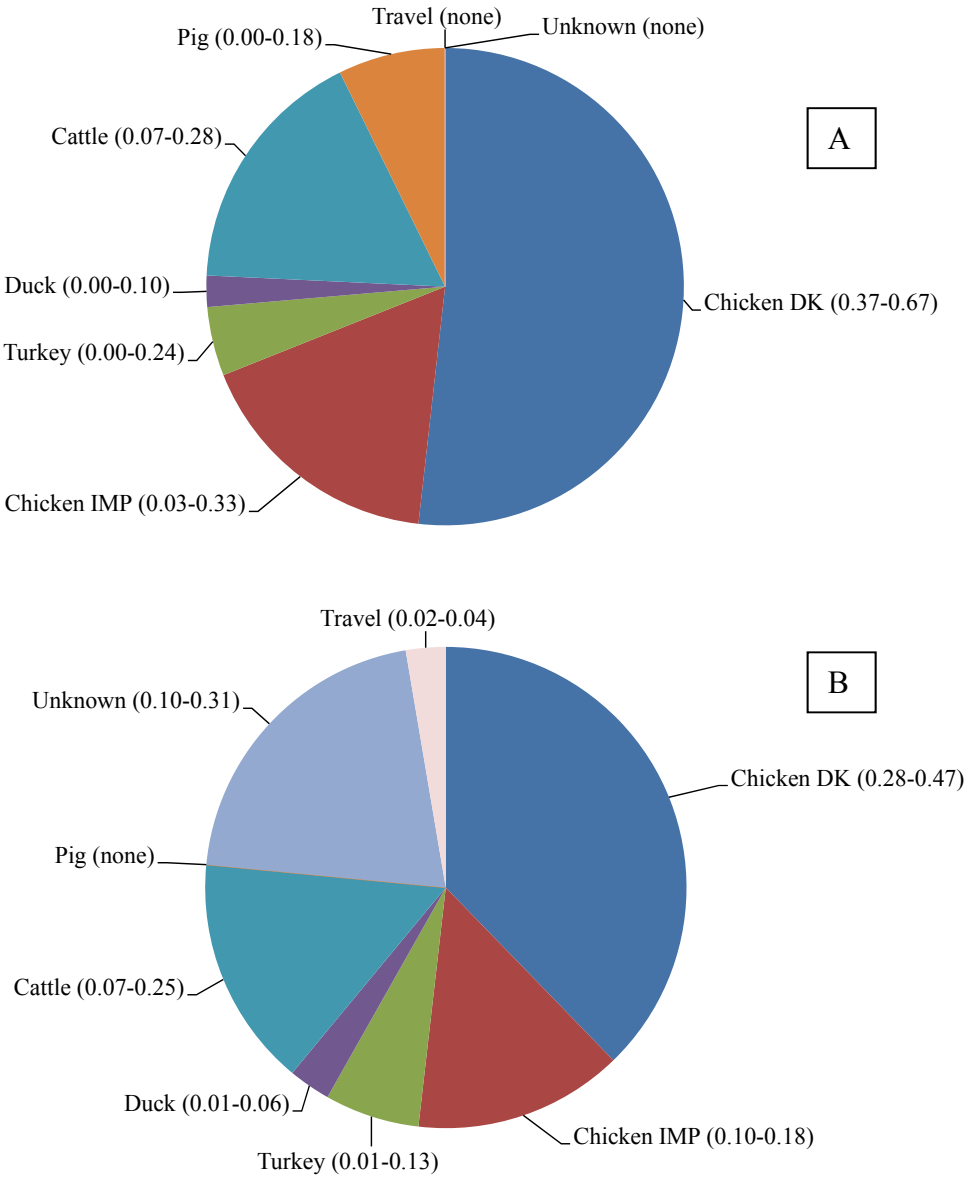

Figure 9. Proportion of cases attributable to the specific source modelled with the basic models, based on MLST (domestic cases and cases without travel history) A) AI model estimating mean proportion and 95% confidence intervals and B) CAMSA model estimating mean proportion and 95% credibility interval

In addition to the application of the basic model, where the AI model only infers source estimates from the included sources (as it was illustrated in Figure 9), the inclusion of travel as a "source" only changed the attribution of cases very little. The vast majority of cases were still attributed to broiler chicken and cattle, and approximately 11% (CI 1-29%) of cases with unknown travel history was attributed to travel; 2% of all apportioned cases. This estimate corresponded to the estimate inferred from the CAMSA model (3%, CI 2-4%).

For the CAMSA model, the sequence type dependent factor (q_i) was fairly equal between STs. Only the estimate for one ST (ST 4811) tended to be higher than the rest, indicating this type to result in relatively more human cases as compared to the other STs. Human case isolates of this type (ST 4811) was mainly domestic and predominantly found in Danish chicken. The q-values (q_i) are depicted in Appendix figure 3. The food related factor (a_j) for cattle tended to be higher compared to the food related factor for other sources (not illustrated). By plotting the observed number of cases (o_i) against the expected number of cases (λ_i) for individual sequence types it seemed that the CAMSA model slightly underestimated the number of cases (illustrated in Appendix figure 4).

8.3.1 Comparison of results

The AI model and the CAMSA model produced similar and robust results. Both models agreed in recognizing broiler chicken as the primary source of human campylobacteriosis; estimating this reservoir to account for over 50% of the human cases. This agrees with the original hypothesis of chicken being the most important single source of human campylobacteriosis. The AI model estimated approximately 69% of cases apportioned to this reservoir against an estimate of 52% of the CAMSA model. A higher proportion of cases were apportioned to Danish chicken compared to imported chicken; 52% and 38% from the AI model and CAMSA model, respectively. The lower estimate from the CAMSA model may, in part, be explained by the proportion of cases which are not attributed to a specified source (the group of unknowns). In general, the estimates of uncertainty around source mean estimates ranged more widely with the AI model compared to the CAMSA model. Uncertainty estimates around all source mean estimates for the two models overlapped. Thus, the proportion of cases attributable to each source was not significantly different.

A Danish case control study estimated fresh chilled chicken to be the associated source for 24% (CI 8-53%) of domestically acquired campylobacteriosis cases *(123)*. Further, the Scientific Opinion on Quantification of the risk posed by broiler meat to human campylobacteriosis in the EU, published by EFSA, suggests that handling, preparation and consumption of broiler meat account for only 20-30% of human cases, while 50-80% may be attributed to the broiler chicken reservoir as a whole *(41)*. As the Danish source attribution studies were carried out using models linking human cases to the reservoir of origin, it is not possible to infer the proportion of cases caused by handling, preparation and consumption of Danish chicken meat, because the isolates collected represent all transmission routes from the chicken reservoir to the consumer. This is in contrast to the imported chicken meat, where transmissions routes prior to packaging are of no risk for the Danes. Thus, with an estimate of the ratio of Danish/imported meat available for sale (60/40 in 2008) and assuming no difference in ability to induce illness between types; a combination of source estimates and consumption data, could approximate the Danish chicken meat to be the associated vehicle in 21% of all domestically acquired cases (calculation based on the CAMSA model estimate).

Cattle was found to be the second most important source. High *C. jejuni* prevalence has been reported in cattle *(3, 4)*, however very low occurrence has been found in beef *(3, 4)*. If cattle should bear the second highest responsibility in relation to human campylobacteriosis, routes other than

meat should be considered. This would agree with the results from a Dutch comparative exposure assessment which ranks farm animal contact higher than beef with regard to importance of transmission *(43)*.

The *Campylobacter* occurrence in meat from ducks and turkeys is high in Denmark (national surveillance data, not published). Only few cases were attributed to the duck reservoir. There may be several reasons for this. A large proportion of isolates from duck meat were only found to a lower degree in humans, and maybe the STs in duck are less prone to induce human illness. Another reason that may influence the result is probably related to the common way of handling and preparing this product, and to the fact that much smaller amounts of duck meat is consumed compared to chicken meat. Traditionally, ducks are prepared whole and long before the garnish. Accordingly, the risk of cross-contamination is low. The proportion of cases attributed to turkey was smaller than broiler chicken and higher than duck. In Denmark, turkey is mainly bought as meat cuts rather than whole turkeys and handling resembles that of broiler meat rather than that of duck. Also, the proportion of turkey meat available for sale is approximately one sixth of broiler meat why the importance of this reservoir was also expected to be lower (given equal infection potential of types). Finally, duck meat is primarily sold frozen, while turkey meat sold as chilled. The freezing reduces concentration and thereby also the risk.

Estimates of the importance of the pig reservoir were small. This agrees with surveillance data, which infers the prevalence of *Campylobacter* spp. in pork to be very low (0.2% in 2002) and the proportion of *C. jejuni* in the reservoir as a whole being inferior to *C. coli*. The source estimate might have been different if additional isolates could have been included; though, the estimate would still be expected to be small.

Other studies have observed that campylobacteriosis cases involving children living in rural areas are more frequently associated with the ruminant reservoir as opposed to other groups *(82, 115)*. At present, spatial and demographic factors have not been included in the Danish study, but it could be interesting to explore this aspect.

8.3.2 Adding discriminatory power

The potential in adding another discriminating attribute, in this case the sequenced *flaA* gene, was explored. In general, the inclusion of the *flaA* gene did not cause considerable changes in the results of the modelling, thus no changes in the importance of sources were observed.

For the CAMSA model, this addition decreased the number of cases that the model was able to assign to a source. This model is seeking exact matches between human and source types, meaning that adding discriminatory power results in fewer matches between cases and sources. The proportion of cases attributed to the different sources decreased in approximately equal magnitude, suggesting that additional sampling of sources are needed to cover the large variation in STs. Only the proportion assigned to cattle decreased slightly more.

Results from the AI model, after inclusion of the *flaA* gene, increased the proportion of cases attributed to chicken at the expense of the proportion attributable to cattle. The uncertainty around the attribution estimates still ranged widely, but diminished slightly following the inclusion of *flaA*. The inclusion of *flaA*, resulted in a larger proportion of cases more strictly associated with chicken (illustrated in manuscript VI).

For the CAMSA model a larger proportion of cases fell in the group "Unknown" including a proportion from each group except travel. The proportion of cases attributed to cattle decreased slightly more compared to the other groups, which is in alignment with the findings of the AI model.

Comparison of results from both models based on the Basic models and models including the *flaA* gene are presented in Figure 10.

MLST is highly discriminatory for *C. jejuni*; however, STs are not source specific. Therefore the source attribution would benefit from identification of a source specific feature. By addition of the *flaA* type it seemed that the apportioning of cases between the broiler chicken reservoir and the cattle reservoir was slightly improved.

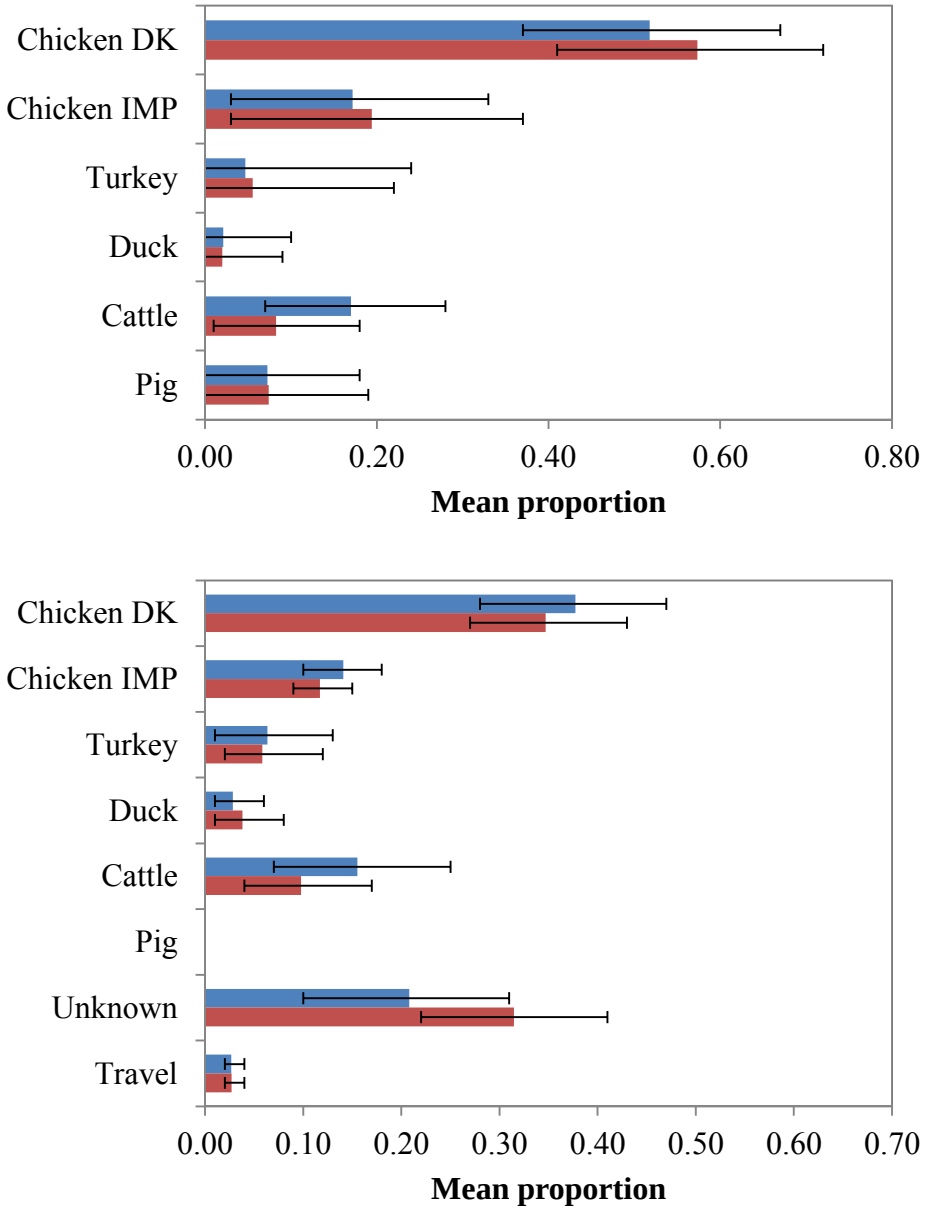

Figure 10. Comparison of results from the Basic models and models including the *flaA* gene; the AI model (A) and the CAMSA model (B). Proportion of cases attributed with the Basic model (blue) and the Basic model+*flaA* (red). Mean proportion of cases attributed to each source with 95% credibly intervals.

8.3.3 Are data representative?

It was assumed that the collection of isolates from all over the country covered geographical variation. However, the great heterogeneity of *Campylobacter* complicates the coverage of the full genetic diversity. To achieve the best possible attribution, the variation in STs within sources

should be covered. However, according to rarefaction analysis, this was not the case with the available data (Jonas Larsson, SSI, personal communication). Only the isolates comprised within the cattle reservoir verged on covering the variation in STs.

An additional large number of samples would be needed to cover the huge variety in STs because of the great heterogeneity in *Campylobacter,* which would require an even larger number of samples to be collected. This work would be extremely costly.

Furthermore, during sampling, only one isolate per sample was obtained. However, several types can be present in e.g. the same broiler chicken *(109)*. The occurrence of types will probably differ and by only picking one isolate per sample, statistically, it is most probable that the type of greatest abundance will be collected. Though, it may be that the type that poses the greatest risk for human illness is not the predominant flora and therefore underestimated within the reservoir. The great variation of types even within animals may further complicate the source attribution for *Campylobacter*.

8.3.4 Strength and weaknesses of the Danish source attribution

The MLST approach, which is used as the basic mean for characterization of isolates, is based on typing of seven conserved housekeeping genes. One of the corner-stones in using MLST for source tracking is that these genes are less prone to changes, which would obliterate the association between isolates found in humans and in sources. For example, changes in the *fla*A type have been registered after colonization of chickens for up to just 21 days *(54)*.

A limitation of the microbial subtyping approach is that it is restricted to pathogens that are heterogeneously distributed among the sources, a requirement which is not strictly met by *Campylobacter*. Furthermore, the microbial subtyping approach will only attribute illness to those sources from which isolates are available. A representation of more sources in the model may aid a subdivision of the presently included sources.

As the STs are widespread between reservoirs, the results of the models might have been different if the collected samples from petting zoo goats, water, meat of lamb, milk cattle, etc. had not been lost. The relatively large proportion of cases associated with the cattle reservoir may actually comprise other sources not included in the model at present; e.g. sheep, goats or environmental origin. It may be noted that other studies have found that campylobacteriosis in children below the age of five, especially living in rural areas, are more frequently associated with ruminant sources *(82, 115)*. The proportion of cases attributed to cattle with the present model may include a proportionate large group of children; however, this is to be explored prospectively.

From AMOVA analysis, statistical difference was found for the genetic differentiation between Danish broilers and Danish broiler meat. If this is due to the great genetic variation not being covered by the sampling or caused by recombination, which has been shown to be pronounced even in short term *(54)* is not readily explained. However, if similarity is limited even

within the same reservoir one could speculate if the inferring of source estimates for human cases is reasonable.

The AI model has the advantage of considering the relatedness of isolates, taking estimates of recombination, mutation and migration into account. Considering the substantial genetic variation of *Campylobacter* this may be considered a strength of this model. The model estimates the probability of for each human case to belong to each implicated source; and estimates the total proportion of cases attributable to these sources *(122)*. Accordingly, the inclusion of all putative sources is stressed.

The genetic diversity of *Campylobacter* is a challenge for the CAMSA model, as the apportioning of cases is based on exact match between subtypes in sources and humans. However, the possibility of cases being attributed to a category of 'unknown' allows for the human cases not to fit within the included sources; rendering the possibility of reservoirs not being represented.

One model does not outperform the other but they rather supplement each other. The attribution of both models would benefit from even larger data grounds; including more data concerning the genetic variation.

The transmission routes for *Campylobacter* from reservoir to human exposure are various. The models using subtyping are reservoir models, assigning cases in relation to their supposed origin and do not provide any disclosure regarding the specific transmission routes. In other words, the models can point out the most important reservoir for human infections, but specific problem areas e.g. along the food-production chain cannot be targeted. However, if cost-effective control of the pathogen can be established at the reservoir level, this will resonate to the implicated routes of transmission.

It could be discussed whether to do a comparative exposure assessment to elucidate specific areas of concern. Though, this discipline requires an immense amount of data which is not readily available in Denmark. However, Evers *et al*. *(43)* attempted to estimate the importance of different transmission routes. As data may be difficult to obtain for all relevant sources, estimates from expert opinions or data from other countries may be used, but this may influence results more than intended. Furthermore, using estimates of the mean exposure rather than a distribution, including the variability, may not be a valid measure as it is probably not the mean exposures that pose a human health problem, but rather the single occurrences of high exposure, accordingly, the variability of the estimates may prove to be of great importance *(86)*.

Nonetheless, to the extent data allows, it could be interesting to try to combine the discipline of comparative exposure assessment and the microbial subtype approach. As *C. jejuni* STs are so widespread over sources, it could be interesting to aid the microbial subtype approach by adjusting the importance of reservoirs based on subtyping according to the specific exposure.

9. GENERIC DISCUSSION

The registered number of human campylobacteriosis cases decreased coincidently with the implementation of the first Danish action plans against *Campylobacter* (manuscript I); being significantly lower in the period after the implementation of action plans (2003-2010) compared to the period before (2001-2002). Broiler flock prevalence decreased in the period 2002-2004, and hereafter remained steady (manuscript I), and the risk from imported chilled meat has decreased considerably since 2005 (manuscript V). Drops in human campylobacteriosis have also been seen after implementation of control measures in the broiler production in Iceland and New Zealand *(110, 117)*.

The registered number of human campylobacteriosis cases was lower in the period after the implementation of action plans (2003-2010) compared to the period before (2001-2002). Though, the relative risk in total from broiler meat available for sale in Denmark was approximately the same in the last part of the study period compared to the first period before implementation of the action plans. Evaluation of control measures in the broiler production is traditionally evaluated according to prevalence or the direct human impact through the food chain "from farm to fork". Though, it should be noticed that interventions in the primary production may affect more transmission routes than the evident through the food chain, while interventions in processing are limited to only affect the specific production.

QMRA was used to evaluate the implemented intervention strategies according to estimates of human risk as opposed to the traditional way using prevalence. The use of QMRA proved to be of added value. The main purpose of implementing control strategies was to decrease human risk. The approach of combining prevalence, mean concentration and also the variability of the mean concentration is important in relation to the probability of inducing human illness. Using QMRA was found to be a more accurate method for evaluation of the results compared to an evaluation in terms of prevalence or mean concentrations alone (manuscript V).

The key elements of the Danish *Campylobacter* strategy are biosecurity, scheduling of positive broiler flocks for freezing, and consumer campaigns. Strict compliance with biosecurity measures has been deemed an essential element in the *Campylobacter* control to reduce broiler flock prevalence and maintaining a low baseline *(33)*, while scheduling has proven to produce products that pose a lower risk to consumers, when compared to chilled products *(33)* (manuscript V). It has been concluded, that consumer education has only little effect with regard to risk reduction *(89)*. This regards the education of adults, and may be due to difficulties in changing established behaviour; e.g. in the kitchen. Hence, in Denmark, programs targeting school children have been launched with the aim of teaching kitchen hygiene *(102)*. The effect of interventions at consumer level may be questionable; however, a continued consumer education may be in order to emphasize a common responsibility in reducing the risk of becoming ill.

The Danish action plans are voluntary and strict consistency in compliance with interventions cannot be assumed. Especially in the warmer period of the year, where the *Campylobacter* prevalence in broiler flocks increases, the scheduling of meat from positive flocks is challenging

and with increasing production of special products (chilled), the scheduling becomes even more complicated. Extensive research of techniques to reduce *Campylobacter* numbers on broiler carcasses has been carried out *(33)*. So far, no "silver bullet" has been identified for controlling this pathogen. *Campylobacter* remains the pathogen most frequently associated with human bacterial gastroenteritis in Denmark *(12)* and within the EU *(42)*. Accordingly, a change in strategy to control this pathogen may be necessary. For example, more attention could be dedicated to further mitigation within the primary production to reduce prevalence (e.g. by implementation of fly screens[13]), development of 'multiple hurdle' strategies to reduce the concentration at slaughter, and/or a change of tactics regarding incentives for producers to reduce the occurrence.

To achieve an even higher impact on combating *Campylobacter*, the following points could be considered by risk managers when deciding upon future action plans:

- Intensified biosecurity with fly screening of broiler houses
- Making action plans mandatory
- Providing an economic incentive (benefit or consequence)
- Setting targets/microbiological criteria (mandatory or consented between authorities and industry)
- Optimized processing hygiene
- Changing tactics concerning interventions at slaughter; 'multiple hurdle' strategy
- Investigating interventions against *Campylobacter* in other sources than broilers
- Disclosure of transmission routes

Mounting fly screens on broiler houses can effectively reduce broiler flock colonization in summer in Denmark *(52, 98)*, and would most likely be translated into similar prevalence reduction for broiler meat (manuscript II). One of the issues with the application of fly screens as well as other interventions in the broiler production is the economic issue. The willingness to implement interventions could potentially be improved, if farmers had an economic incentive to do so, as opposed to being imposed with increased costs for implementing interventions. The large Danish companies reward broiler farmers economically for compliance with the code of practise and for producing *Campylobacter* negative broiler flocks (manuscript I). However, the magnitude of the reward is limited and cannot finance larger investments. Still, this strategy increased the awareness of complying with the industry code of practice and keeping a high level of biosecurity.

The nature of the Danish action plans, as a voluntary initiative, may be challenged by making them mandatory, as is the case with freezing of meat from positive flocks in Norway *(66)* and Island *(117)*. However, this approach may not be practically applicable under Danish conditions, given the large proportion of broiler meat being imported. Restricting the Danish production of chilled meat will most likely increase the import of chilled meat to satisfy the demand for chilled meat rather than restricting the availability. Accordingly, the human risk would not change as the risk from

[13] Assessment of the applicability of fly screens in different European countries is within the scope of the international research project CamCon *(64)*.

imported chilled meat, historically, have been higher than or resembling the risk from Danish meat (manuscript V). Enforcing mandatory scheduling of meat from positive flocks would likely have the same effect.

Another way of creating an incentive for producers to continually focus on the improvement in processing practices and hygiene in the production is to establish performance targets. The adoption of performance targets in the sense of microbiological criteria (MC) is available within EU legislation[14]. MC for foodstuffs introduces two different types of targets: Food Safety Criteria (FSC) and Process Hygiene Criteria (PHC). MC sets indicative contamination values above which corrective actions are required in order to maintain the hygiene of the process in compliance with this regulation *(33)*. Targets may be set at all relevant points in the production. As the true proportion of human campylobacteriosis cases associated with broiler meat has yet to be disclosed, setting a performance target for reduction for human cases, to create an incentive for the broiler producing industry, is not appropriate. The effect of implementing MC depends very much on the level of compliance. The reduction of human risk following the implementation of and compliance with e.g. a common MC within the EU is small in countries with a low baseline, i.e. low levels of *Campylobacter* per carcass (associated low level of risk) compared to countries with a high baseline *(33)*. Performance targets have been adopted in the UK and in New Zealand. In the UK, a voluntary FSC was adopted with the UK strategy for *Campylobacter* 2010-2015. The aim is to reduce the percentage of broiler carcasses produced in UK slaughterhouses with the highest level of contamination (measured post chill). From a baseline of 28% of samples with more than 1,000 cfu/g (determined in the EU baseline survey in 2008) targets have been set for reducing the baseline to 19% in 2013 and 10% in 2015 *(9, 10)*. The target was agreed upon by the major stakeholders (The Food Standards Agency, Defra, the UK poultry industry, and major retailers). In New Zealand, they have implemented a mandatory PHC at the slaughterhouse of 3.78 log cfu/carcass on exit from the immersion chiller. Both industry and regulators expressed that a key point in the New Zealand's strategy was the setting of a mandatory *Campylobacter* spp. performance targets (rather than mandating specific interventions) *(110)*. Setting performance targets has not yet been applied in Denmark. The benefit of setting a PHC in Denmark as an incentive to improve processing practices could be explored. According to data obtained from the EU baseline study in 2008, the additional risk reduction would be small when compared to other countries given a common EU MC (under the assumption of complete compliance) *(33)*. Though, more restrictive national levels can be set, but that would likely lead to increased import as a consequence of increased production costs and increased marked prizes for national products. The potential benefit of implementation should be weighed against factors such as economy, feasibility, technical and organizational practicality.

Processing hygiene, with specific focus on *Campylobacter*, has not been prioritized beyond standard GHP principles at slaughterhouse level in Denmark. Though, stringent application of GHP e.g. with respect to accepted spillage of intestinal content during evisceration, may lead to less contamination of broiler carcasses due to potentially reduced cross-contamination (manuscript III). Recent studies propose an indicative relation between *Campylobacter* contamination and lack of

[14] Regulation (EC) No 2073/2005

process control and effective GHP *(50, 106)*. Introduction of MC could provide incentive for increased attention and induce improvement of processing practices *(33)*.

In spite of extensive research concerning decontamination techniques, physical and chemical, for application at the slaughterhouse, no management solution, within legislation, has been identified to solve the *Campylobacter* problem. The optimal control measure should be cheap, effective, not changing the product characteristics of fresh meat, and accepted by consumers. As previously discussed, mandatory freezing is not an optimal intervention under Danish conditions, as it would lead to increased import of chilled meat that has a similar or higher level of *Campylobacter*, compared to the chilled Danish meat, even though the effectiveness in relation to human risk reduction have been reported as considerable *(33, 59)*. Chemical decontamination appears to be more cost-effective compared to freezing *(59)*; though, at present no chemical substances have been approved within the EU. Furthermore, indications of consumer reluctance towards chemically decontaminated products in Denmark have been reported *(73)*. A Danish study, using focus groups and interview studies, revealed that with the exception of hygiene practices, the public is generally skeptical to risk reduction strategies such as decontamination, unless the method is familiar from home-cooking traditions, e.g. freezing and heat treatment *(73)*.

To obtain an increased reduction of *Campylobacter* numbers on the meat, a strategy of using 'multiple hurdles' may be used. Applying several control measures in sequence may create a synergetic decontamination effect. The efficacy of the 'multiple hurdle' approach has not been documented with regard to *Campylobacter* in the broiler production. However, the approach of multiple-sequential interventions for decontamination purposes has been shown to be an effective way of reducing microbiological contamination of beef carcasses *(16)*. Further studies within this area could be an important new addition in the control of *Campylobacter*.

At EU level, handling and consumption of broiler chicken meat has been estimated to account for 20-30% of human campylobacteriosis cases, while 50-80% of cases may be associated to the reservoir as a whole *(41)*. Results of the Danish source attribution work showed that the vast majority of domestically acquired human campylobacteriosis cases were apportioned to the broiler chicken reservoir, while the second most important reservoir was cattle (manuscript VI). A significantly larger proportion of cases were attributed to the Danish broiler chicken reservoir compared to imported chicken (based on samples collected in the period 2007-2008). This observation does in part agree with the risk estimates obtained from the assessment of risk of Danish and imported broiler meat available for sale in Denmark. In 2008, the risk from Danish and imported meat was fairly similar, however in 2007 and 2009-2010, the Danish broiler meat was estimated to pose a larger relative risk compared to imported meat, when including sales data (Appendix table 3). Furthermore, the source attribution is a reservoir model, while the risk estimates is inferred from exposure estimates from the meat transmission route only. As the source attribution modelling works at a reservoir level, source estimates reflect cases associated with handling and consumption of meat but also animal contact and environmental transmission. The Danish broiler chicken reservoir comprises more transmission routes compared to imported chicken, as transmission routes prior to packaging for the imported meat are of no risk to the Danes. This may

in part also affect the attribution of cases to the cattle reservoir, which also comprised samples from the Danish production as oppose to samples of turkey and duck, which originated primarily from imported products (as the turkey and duck meat production in Denmark is considered to be inferior, compared to the proportion of meat being imported for human consumption). Accordingly, from the source attribution modelling, the reservoirs of Danish broiler chicken and cattle were expected to be overrepresented compared to imported broiler chicken, turkey and duck. Though the exact proportion, however, is not known.

In Denmark, control strategies are in place for broilers and broiler meat, and optimization of these strategies could be discussed with regard to adoption of more control measures, increasing the efficacy of existing measures, upping incentives to comply with already implemented measures and/or proceeding with implementation of targets. Whether to implement control measures in other sources could be speculated on. However, the transmission routes of *Campylobacter* are various and elucidation of the most important routes seems to be important prior to the implementation of extensive and expensive action plans. Furthermore, approximately one third of all campylobacteriosis cases are considered to be travel related. It might be worthwhile to target travelers going to countries which have been associated with an elevated risk of infection.

10. CONCLUSION

Danish action plans to control *Campylobacter* in broilers and broiler meat comprise initiatives covering the whole domestic food chain "from farm to fork". Initiatives involve biosecurity in the primary production; scheduling of *Campylobacter* negative flocks to production of chilled meat and positive flocks to the production of frozen products, to the extent possible, and consumer campaigns to reduce cross-contamination in domestic kitchens (manuscript I). In addition, the case-by-case control was introduced in 2007; targeting high-risk batches of fresh meat of both Danish and imported origin. With the implementation of the first Danish initiatives and action plans against *Campylobacter*, the prevalence decreased in the Danish broilers from 2002 to 2004 as well as in chilled broiler meat at slaughter, 2004-2006 (manuscript I).

The *Campylobacter* prevalence in Danish broiler flocks was found to be a strong predictor for the seasonal occurrence of *Campylobacter* in Danish chilled broiler meat. Seasonality was more distinct for chilled meat compared to frozen meat, and was most pronounced for Danish meat compared to imported meat (manuscript II).

The decontamination techniques; forced air chilling, crust freezing and steam-ultrasound investigated on-plant, resulted in reductions of the concentration of naturally occurring *Campylobacter* spp. on carcasses and fillets, with some variation in the reduction achieved. Mean reductions of 0.44, 0.42, and ≥2.51 log units were obtained by forced air chilling, crust freezing, and steam-ultrasound treatment, respectively. The steam-ultrasound method was the most effective decontamination method; though, an adverse effect of this technique was a slightly boiled appearance of the carcass skin. Visceral rupture yielded an increase in *Campylobacter* of 0.9 log units. When used alone, all methods reduced the *Campylobacter* concentration. It could be of interest, though, to investigate if a combined use of the methods would result in an additional reduction. No method performed equally to freezing when considering reductions in *Campylobacter* counts against adverse effects (manuscript III).

Log reductions obtained by decontamination, physical (freezing for 24 h and 7 d) or chemical (tartaric acid and TSP), in laboratory scale with inoculated meat medallions, tended to be unaffected by the initial *Campylobacter* concentration on the meat, with the exception of reductions obtained by freezing (for seven days) and treatment with TSP (marginally significant). For these treatments, analysis suggested that different reductions were obtained using high inoculation levels (10^7 cfu/sample) compared to the lower levels (10^3-10^5 cfu/sample). All results were significantly influenced by strain. Accordingly, reductions obtained in studies with high concentrations of *C. jejuni* and one or few *C. jejuni* strains may not represent the general result for the species (manuscript IV).

The human risk associated with the four main categories of broiler meat available for sale at retail in Denmark (Danish and imported, chilled and frozen meat) was assessed by QMRA. Human risk was generally higher from chilled meat compared to frozen meat. The most evident changes were the decreasing risk from chilled imported meat (2005-2010) and the increasing risk from Danish frozen meat (2003-2010). No marked changes in human risk from Danish produced, chilled

broiler meat and imported frozen meat were observed. The reduction of the human risk from imported chilled meat coincides to great extent with the implementation of the case-by-case control. Accordingly, the case-by-case initiative may have provided the retailers with an incentive to heighten standards for their suppliers. The increasing risk from Danish produced, frozen broiler meat coincided with the control measure of scheduling of meat from *Campylobacter* positive broiler flocks to frozen production to the extent possible. The allocation of meat from positive flocks results in a frozen production with a higher *Campylobacter* prevalence. Even though the frozen meat is *Campylobacter* positive, it still constitutes a lower risk to the consumer compared to chilled meat, as freezing reduces the *Campylobacter* numbers on the meat. The registered number of human campylobacteriosis cases was lower in the period after the implementation of action plans (2003-2010) compared to the period before (2001-2002); though the relative risk in total from broiler meat available for sale in Denmark was approximately the same in the last part of the study period compared to the initial period before implementation of the action plans.

The use of QMRA in the evaluation of intervention strategies proved to be of added value, compared to using prevalence alone. The approach of combining prevalence, mean concentration and also the variability of the mean concentration was important in relation to performing the evaluation more accurately in relation to a human health perspective (manuscript V).

The source attribution models, the AI model and the CAMSA model produced similar results based on subtyping with MLST. Both models attributed the vast majority of cases (>50%) to the broiler chicken reservoir, while the second most important reservoir was cattle (mean estimate 16-17%, the CAMSA model and AI model, respectively). Credibility intervals (95%) around the mean source estimates for the two models overlapped; hence, mean estimates for each source computed by the two models were not significantly different (manuscript VI).

Addition of the *flaA* gene to the MLST sequence type added slightly more discriminatory power to the modelling. The apportioning of cases between the broiler chicken and the cattle reservoir were slightly more specific. The source estimate based on MLST+*flaA* type increased slightly for the broiler chicken reservoir at the expense of the cattle reservoir, compared to attribution based on MLST alone (manuscript VI).

The present PhD study has provided results which are useful for future risk management decisions regarding the control of *Campylobacter* in the broiler production, and in which reservoirs to target interventions. The PhD study has undertaken the evaluation of the action plans against *Campylobacter* in Denmark based on extensive national surveillance data; focused on the control of this pathogen on broiler meat at slaughter, the human risk from broiler meat and disclosure of sources associated with human cases (sporadic and domestically acquired). The Danish strategy to control *Campylobacter* has had a positive effect; reducing the prevalence in broiler flocks, producing lower risk products with scheduled freezing, and reducing risk from imported, chilled meat. A small decrease in the number of human cases has also been observed.

11. PERSPECTIVE

How to proceed from this work?

Suggestions for additional control options, which could be considered for future action plans against *Campylobacter*, are the mounting of fly screens on broiler houses which has been shown to effectively reduce broiler flock colonization in summer in Denmark *(52, 98)*. This would most likely be translated into similar prevalence reduction for broiler meat and potentially also affect other of the transmission routes from this reservoir. Intervention strategies targeting *Campylobacter* at slaughter could involve increased attention towards hygienic processing and optimization of the reduction of *Campylobacter* numbers on carcasses by using 'multiple hurdle' strategies. Finally, the potential benefits of implementing microbiological criteria could be explored; weighting benefits against factors concerning economy, feasibility, technical and organizational practicality.

Prospectively, it would be interesting to compare the human risk from Danish meat sampled at retail with the risk from meat sampled at the plant to assess the importance of the bias caused by overrepresentation of the smaller slaughterhouses in the retail sampling. Future evaluation of control strategies would benefit from using risk estimates inferred from a combination of prevalence, concentration and variability around the mean concentration, e.g. by QMRA. Further, it would be of great interest to compute uncertainty around the risk estimates.

With regard to source attribution, it would be of interest to perform an indebt analysis of the source attribution data, involving increased focus on epidemiological aspects such as the source distribution among age groups and among people living in rural versus urban areas.

Furthermore, the transmission routes from the broiler chicken reservoir to humans, other than handling, preparation and consumption of broiler meat, are not well understood. Disclosure of these routes could possibly aid the understanding of the epidemiology within this reservoir, and generate knowledge on how we can be able to target this important reservoir in even broader perspective.

Source attribution for *Campylobacter* by the microbial subtyping approach is suffering from the heterogeneous distribution of types between reservoirs. An increased credibility in apportioning of cases could be obtained by identification of a source specific attribute, which would add the appropriate discriminatory power. Though, such a factor is still to be identified. Alternatively, it could be interesting to explore the combination of source attribution and comparative exposure assessment, and hereby adding information of the plausibility of *Campylobacter* reaching the consumer via the various transmission routes.

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13. MANUSCRIPTS

Manuscript I: **Danish strategies to control *Campylobacter* in broilers and broiler meat: facts and effects.**

Manuscript II: **Seasonal influence on the prevalence of thermotolerant *Campylobacter* in retail broiler meat in Denmark.**

Manuscript III: **Reduction of thermotolerant *Campylobacter* species on broiler carcasses following physical decontamination at slaughter.**

Manuscript IV: **Effects of decontamination at varying contamination levels of *Campylobacter jejuni* on broiler meat.**

Manuscript V: **Human risk from thermotolerant *Campylobacter* on broiler meat in Denmark.**

Manuscript VI: **Source attribution of human campylobacteriosis in Denmark**

Manuscript I

Danish strategies to control *Campylobacter* in broilers and broiler meat: facts and effects

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Danish strategies to control *Campylobacter* in broilers and broiler meat: facts and effects

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SUMMARY

Thermotolerant *Campylobacter* spp. have been the most common bacterial cause of human gastrointestinal disease in Denmark since 1999. In 2003, the Danish voluntary strategy to control *Campylobacter* was intensified. The focus was on biosecurity, allocation of meat from *Campylobacter*-negative broilers to the production of chilled products, and consumer information campaigns. From 2002 to 2007, the percentage of *Campylobacter*-positive broiler flocks at slaughter decreased from 43% to 27%. After processing, *Campylobacter*-positive samples of chilled broiler meat fell from 18% in 2004 to 8% in 2007. Furthermore, the number of registered human *Campylobacter* cases decreased by 12%; from 4379 cases in 2002 to 3865 cases in 2007. We believe that the observed decrease in the occurrence of *Campylobacter* in broilers and broiler meat and the coincidental fall in the number of registered human cases is, in part, a result of the implemented control strategy.

Key words: Action plan, broiler meat, broilers, campylobacteriosis, monitoring.

INTRODUCTION

Thermotolerant *Campylobacter*, primarily *Campylobacter jejuni* and *Campylobacter coli*, are the most common bacterial causes of human gastrointestinal disease in Denmark [1], EU [2] and many other industrialized countries worldwide [3]. Generally, the increase in human *Campylobacter* cases started in the 1990s, and in many countries the incidence continues to increase [3]. It is generally accepted that poultry meat is the most important source of foodborne *Campylobacter* infections [3]. In Denmark, fresh, chilled broiler meat has been identified as a major risk factor of sporadic campylobacteriosis [4].

The first Danish initiatives to combat *Campylobacter* were initiated in the 1990s, primarily by the poultry industry and the Danish food authorities and comprised hygienic measures at farm level as well as initiation of a monitoring programme of broiler flocks and retail food, especially poultry meat. Moreover, consumer information was also included (Table 1). The initiatives were much inspired by work done in Sweden on *Campylobacter* colonization of broiler flocks [5].

In 1998, *Campylobacter* was included in the national governmental pathogen strategy, which was

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Table 1. *The first Danish initiatives to control* Campylobacter

Step	Year of initiation	Initiative	Aim
Primary production	1995	Research in risk factors for flock infection	Prevention of flock infection
	1996	Intensified education of broiler producers in hygiene barriers	Prevention of flock infection
	1998	Higher price for *Campylobacter*-negative broiler flocks	Economic incentive to encourage the implementation of improved hygiene and biosecurity measures
	1998	Monitoring of the *Campylobacter* status of all broiler flocks	In the beginning as part of the risk-factor studies. Later, for implementation of logistic slaughter (=the slaughter of negative birds before positive ones)
	2001	Development of PCR method for rapid testing of flocks	Improved analysis and thereby faster identification of flock status
Processing	2002	Testing of process operations	Knowledge for future management decisions
Retail	1995	Monitoring of *Campylobacter* in retail food, especially poultry meat	Identification of food sources important for human exposure to *Campylobacter*
	2000	Production and launch of a *Campylobacter*-free frozen broiler	Increased food safety
	2000	Development of semi-quantitative and quantitative methods for enumeration of thermotolerant *Campylobacter* in food	Quantification of the contamination
Consumer	1994	Consumer information on bacteria present in broiler meat and guidance on kitchen hygiene via consumers' magazines and leaflets in supermarkets	Consumer education
	1999	Leaflets on food hygiene during barbequing and regular press releases	Consumer education

anchored in the principles of Food Safety Risk Analysis [6, 7] and in 1998 a risk profile for pathogenic *Campylobacter* spp. in Denmark was prepared [8]. The risk profile recommended continuing the management process commissioning a quantitative risk assessment of *Campylobacter* in food. Consequently, a quantitative risk assessment of *C. jejuni* in broiler meat was published in 2001 [9]. *C. jejuni* in broiler meat was subjected to assessment, as this pathogen–food combination was identified as the major risk for human *Campylobacter* infection.

Based on the outcome of this risk assessment, national research projects as well as experiences from Iceland [10], a voluntary intervention strategy for *Campylobacter* in broiler production was developed in collaboration between government, non-governmental organizations and the poultry industry in 2003 (Table 2). The intervention strategy comprised initiatives throughout the production chain; primary production, processing and consumers. One of the key elements of the Danish strategy was to reduce numbers of campylobacter on broiler meat [11]. This was based on the Danish risk assessment, which predicted that a 2 $\log_{10}$ reduction of the concentration of *C. jejuni* on the meat could result in a 30-fold decrease in the number of human *Campylobacter* cases (related to consumption of broiler meat) [9]. To obtain a similar reduction of the number of human cases, the prevalence of broiler flocks should be reduced or kitchen hygiene should be improved 30-fold. Based on the experience at that time, it was assumed that a change of that magnitude of flock prevalence and/or kitchen hygiene would be difficult to obtain, whereas it would be relatively easy to implement the intervention of voluntary freezing of colonized broiler flocks, which had already proven effective as an intervention in Iceland [10, 12]. Channelling *Campylobacter*-negative flocks to production of fresh, chilled broiler meat and *Campylobacter*-positive flocks to frozen products, has also been suggested as a promising intervention strategy by several researchers [13–15]. Therefore, it was decided to channel *Campylobacter*-positive broiler flocks to frozen production, as much as possible, as freezing is known to reduce campylobacter counts by about 2 $\log_{10}$ units [12, 16].

Table 2. *Initiatives to control* Campylobacter *in the Danish voluntary control strategy from 2003*

Step	Year of initiation	Initiative	Aim
Primary production	2003	Reinforcement of the compliance with the industry code of practice (hygiene and biosecurity)	Reduction of flock infection
	2003	Increased bonus for *Campylobacter*-negative broiler flocks and/or for compliance with the industrial code of practice	Economic incentive to obtain a reduction of flock infection
	2003	Limitation of partial slaughter and/or research in methods to improve biosecurity during this procedure	Reduction of flock infection
	2003	Research into measures to prevent flock colonization, e.g. insect control, restricted admission; and into methods to reduce the *Campylobacter* concentration in the broilers' gut, e.g. via feed additives or phage therapy	Knowledge for future management decisions
Processing	2002	Channelling of *Campylobacter*-negative flocks to fresh, chilled meat production and *Campylobacter*-positive flocks to frozen production (as much as possible)	Reduce the percentage of *Campylobacter*-contaminated fresh, chilled meat and the concentration of *Campylobacter* in contaminated meat
	2003	Logistic slaughter (=the slaughter of negative birds before the positive ones)	Prevention of cross-contamination from *Campylobacter*-positive to -negative flocks
	2003	Research in critical operations during processing	Knowledge for future management decisions
	2003	Research in methods to reduce numbers of campylobacter on broiler meat	Knowledge for future management decisions
	2004	Monitoring of *Campylobacter* in chilled broiler meat in two major slaughterhouses	Knowledge for risk management
Retail	2003	Promotion of the production and sale of *Campylobacter*-free chilled broilers	Reduce amount of *Campylobacter*-contaminated broiler meat
Consumer	2003	Information campaign aimed specifically at young people focusing on how to handle poultry products in domestic kitchens	Consumer education

In 2008, a new 5-year action plan against *Campylobacter* was launched by the Danish Government (Table 3). This was developed in cooperation between the industry, the Danish Veterinary and Food Administration and the Technical University of Denmark and was guided by recommendations from an Expert Consultation held in Copenhagen in 2007 [15]. The action plan aims at reducing the prevalence of *Campylobacter* in Danish broiler flocks and in Danish broiler meat even further, and innovatively the plan also focuses on reducing the risk of acquiring *Campylobacter* infections from imported broiler meat.

In Denmark, testing for *Campylobacter* in every broiler flock at slaughter has been part of the national monitoring programme since 1998. However, in order to be able to channel the production, in 2003, the two major slaughter companies also initiated a private monitoring programme where all flocks are sampled on farm 7–10 days before slaughter. At retail, broiler meat has been monitored since 1995 and from 2004 onwards samples have also been collected at two large slaughterhouses handling more that 98 % of Danish broilers (Table 2). In addition, campylobacteriosis in humans has been notifiable since 1980. In this paper, we present some of the Danish monitoring data on *Campylobacter* in broilers, broiler meat and humans in order to identify any trends in the occurrence of *Campylobacter*. The main objective of this paper was to explore if any changes coincide with the implementation of intervention initiatives in the broiler production chain from farm to fork.

MATERIALS AND METHODS

Campylobacter in broiler flocks

Since 1998 all Danish broiler flocks have been tested for *Campylobacter* at the point of slaughter. The

Table 3. *The Danish action plan to control* Campylobacter *2008–2012*

Step	Initiative	Aim
Primary production	Development and implementation of an industry code of practice for production hygiene	Increased focus on biosecurity measures
	Development of fly screens for broiler houses	Reduction of *Campylobacter* transmission into broiler houses
	Optimization of ante-mortem sampling	Improvement of flock channelling to chilled and frozen products
	Collection of data and suggestions for risk management of free-range and organic broilers	Identification of possible management options for this branch of production
Processing	Optimization of channelling of broiler flocks	Improvement of flock channelling to chilled and frozen products
	Investigation of applicable methods for decontamination and improved hygiene	Knowledge on reduction methods
	Final testing of steam-ultrasound in-line equipment	Knowledge on reduction methods
	Expansion of the slaughterhouse surveillance to cover all Danish slaughterhouses with production of chilled broiler meat	Expanded surveillance of the chilled, broiler meat production
Retail*	Ongoing development of and intensified case-by-case control	Reduction of risk from highly contaminated broiler meat
Consumer	Information for consumers (general and in relation to barbequing)	Improvement of consumer awareness
	Focus on information on kitchen hygiene for schoolchildren	Early education of the 'new' consumers
	Development of Danish *Campylobacter jejuni* source account	Insight into the sources of human *Campylobacter jejuni* cases

* Samples collected at wholesale.

number of flocks tested has varied from about 6000 in 1998 to about 4500 in 2007 [17]. From 1998 to the end of 2001, 10 cloacal swabs were collected at slaughter; these were pooled and tested using conventional microbiological techniques as described by Wedderkopp *et al*. [18]. From 2002, pooled samples were analysed using the PCR detection method described by Lund *et al*. [19]. In 2002, the major slaughter companies also initiated testing of flocks on farm. Samples were collected as one pair of sock samples on the farm and analysed using the same PCR method as described for cloacal swabs.

Campylobacter on meat after processing

Since 2004, to evaluate the effect of channelling *Campylobacter*-positive flocks to frozen production, samples of fresh, chilled broiler meat have been sampled at two Danish slaughterhouses, processing more than 98% of domestic broilers. In the first two years, 18 samples were collected weekly at each slaughterhouse. For economical reasons, the sample size was reduced to 10–12 samples per week in 2006 and 10–12 samples every other week in 2007. Pieces of broiler meat, cut from the surface of the samples, were analysed for numbers of thermotolerant campylobacter according to a quantitative procedure described by Rosenquist *et al*. [20] based on rinsing of the sample 1:1 (wt/wt) and plating of appropriate 10-fold dilutions onto selective Abeyta–Hunt–Bark (AHB) agar plates with 0·1% triphenyl tetrazolium chloride added for red-staining of colonies. Samples were categorized as negative if campylobacter were not detected (detection limit: <10 c.f.u./g). Conversely, samples were categorized as positive whenever campylobacter were detected.

Campylobacter in human gastrointestinal disease

Data on human infections were obtained from the Danish laboratory surveillance system. Clinical microbiology laboratories (of which there are 14 in Denmark, all public) are required to report all first-positive (i.e. the first diagnosis for any individual within a 6-month period) cases of campylobacteriosis to Statens Serum Institut every week. Foreign and domestically acquired infections can only in part be separated as only a minor subset of notifications

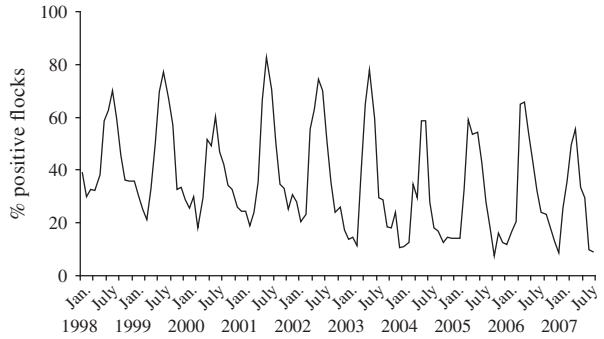

Fig. 1. Prevalence (in percent) of broiler flocks colonized by thermotolerant *Campylobacter* tested by cloacal swabs at slaughter, by month, 1998–2007.

include information about the country of infection. Data are entered into a national database, the Register of Enteric Infections. Although techniques may vary between laboratories, faecal samples from patients suffering from diarrhoea were generally analysed by emulsifying a standardized inoculum in sterile saline and culturing on modified charcoal cefoperazone deoxycholate agar (mCCDA), as described by Engberg *et al*. [21].

Statistics

Analysis of trend was carried out using linear regression analysis in Microsoft Office Excel 2003 and analysis of quantitative data was done by analysis of variance using the statistical software SAS 9.1.3 (SAS Institute Inc., USA). An α-value of 0·05 was considered to be statistically significant in the statistical analyses.

RESULTS

Campylobacter in broiler flocks

Since the interventions were introduced in broiler production, Denmark has experienced an overall decrease in the prevalence of *Campylobacter*-positive broiler flocks at slaughter from 43% in 2002 to 27% in 2007. The prevalence of *Campylobacter* in the broiler flocks at slaughter had a distinct seasonal pattern, with the highest prevalence observed during June–November (up to 78%) and the lowest prevalence observed during December–May (down to 7%) (Fig. 1). For the high-prevalent period the overall prevalence decreased from 55% in 2002 to 39% in 2007. For the low-prevalent period (December–May) the prevalence decreased from 27% in 2002 to 20% in 2003 and has since remained stable at 13–17% (Fig. 2). Thus, for both the high- and the low-prevalent period of the year, *Campylobacter* prevalence has decreased.

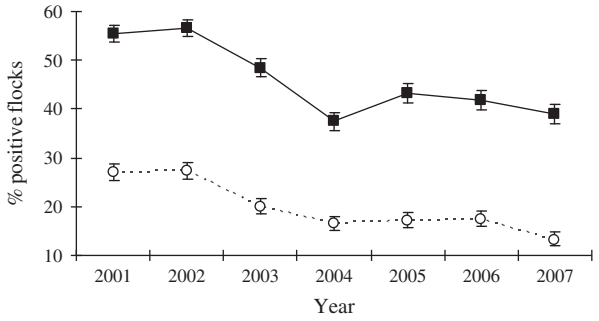

Fig. 2. Prevalence (in percent) of broiler flocks colonized by thermotolerant *Campylobacter* at slaughter, divided into a high-prevalent period (June–November; –■–) and a low-prevalent period (December–May; - -○- -), with 95% confidence intervals, 1998–2007. Prevalence estimation is based on the sum of samples in the particular periods (positives and total).

Campylobacter in meat after processing

The percentages of *Campylobacter*-positive samples of chilled broiler meat collected at two Danish slaughterhouses are presented in Figure 3. The number of *Campylobacter*-positive samples shows a seasonal pattern similar to that of the *Campylobacter* prevalence of broiler flocks. On a weekly basis, fewer high peaks of *Campylobacter*-positive samples were observed in 2005, 2006 and 2007 compared to 2004, indicating a smaller fraction of meat being contaminated. Furthermore, the overall seasonal peak became less pronounced during the monitoring period from 2004 to 2006. In 2004, the total annual percentage of *Campylobacter*-positive samples was 17·8%. This fell to 12·3% in 2005 and fell further to 7·9% in 2006 and 8·1% in 2007. The decreasing trend from 2004 to 2006 is statistically significant ($P = 0{\cdot}042$). In 2007, the occurrence was similar to 2006. In regard to numbers of campylobacter on positive broiler meat samples, the mean concentration showed little variation from 2004 to 2007 ($P > 0{\cdot}05$).

Campylobacter in human gastrointestinal disease

The number of total registered human campylobacteriosis cases in Denmark began to increase in the mid-1990s and peaked at 4620 cases in 2001, corresponding to an incidence of 82·4/100 000 population

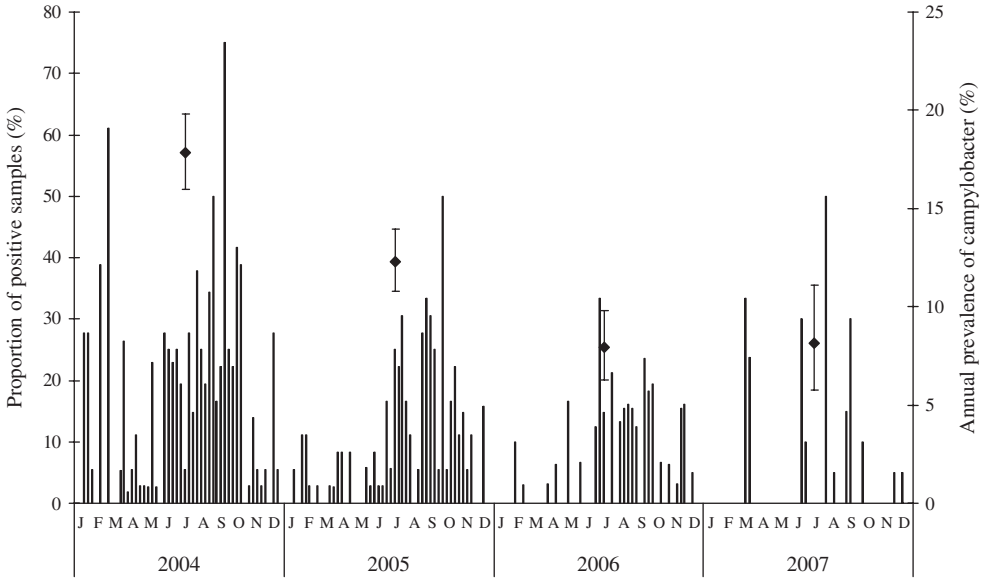

Fig. 3. Weekly mean (columns) and annual (◆) prevalence (in percent) of *Campylobacter*-positive samples of fresh, chilled broiler meat after processing in two Danish slaughterhouses. Data labels indicate 95% confidence intervals for annual prevalence, 2004–2007.

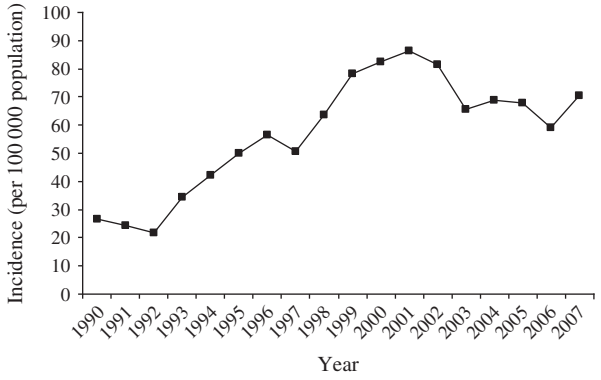

Fig. 4. Incidence of human campylobacteriosis cases in Denmark per 100 000 population, 1990–2006.

(Fig. 4). However, in 2002 the number of cases fell and with some fluctuations has continued to fall; in 2007 a total of 3865 cases were registered, corresponding to an incidence of 70·6. Overall this represents a fall of 12% from 2002 to 2007.

DISCUSSION

At farm level, the Danish initiatives to reduce *Campylobacter* included a number of improvements in biosecurity in and around broiler houses. While it is not possible to identify the effect of each single initiative at the farm, the implementation of the control strategy does coincide with a decrease in the number of positive flocks. Reductions of flock prevalence have also been observed in Sweden, Norway and Iceland, following the implementation of *Campylobacter* control strategies, in which biosecurity has also played a major role [1, 22, 23].

The Danish monitoring of the two major broiler meat producers indicated that the voluntary channelling in these slaughterhouses seemed to improve from 2004 to 2006 due to smaller seasonal fluctuations within years and decreasing *Campylobacter* prevalence for fresh, chilled broiler meat. However, these observations, are probably not only influenced by channelling but also by other voluntary initiatives in the slaughterhouses, e.g. logistic slaughter (the slaughter of negative birds before positive ones) and the implementation of crust-freezing of broiler meat portions. Crust-freezing results in a reduction in numbers of campylobacter of $\sim 0{\cdot}5$ $\log_{10}$ c.f.u. [24], which means that meat containing very low numbers will probably test negative after treatment resulting in a reduction in the overall prevalence. Moreover, logistic slaughter is expected to reduce the overall prevalence, but in regard to reduction in numbers of human cases, logistic slaughter is, by several risk assessments, estimated to have only a negligible effect due to the expected low numbers of campylobacter on the originally negative, but cross-contaminated broiler meat [25].

As previously mentioned, the percentage of meat contaminated with *Campylobacter* decreased from 2004 to 2006 in the two slaughterhouses investigated, although the mean concentration of *Campylobacter* on the positive samples remained unchanged. This does not necessarily reflect that a reduction in the concentration of *Campylobacter* on the meat has not been achieved. Based on the fact that more meat tested negative from 2004 to 2006 in combination with implementation of crust-freezing and logistic slaughter in the slaughterhouses, it is probable that the concentration has been reduced in a proportion of the broiler meat, resulting in the meat testing negative after slaughter.

The observed decrease in the prevalence of broiler flocks and fresh, chilled broiler meat has not translated directly into a considerable drop in the number of human cases. This may be explained by the actual effect being counterbalanced by several factors. First, the broiler meat available at retail in Denmark is not exclusively Danish and nationwide monitoring has shown that *Campylobacter* is found more frequently in imported broiler meat compared to domestically produced broiler meat [17]. Second, the market share of imported broiler meat has increased from about 20% in 2002 to 40% in 2006 [26, 27]. Third, chilled broiler meat from smaller Danish slaughterhouses is also available at retail. The *Campylobacter* status of meat from these slaughterhouses is unknown, but will be monitored in future as part of the new action plan. Finally, since the exact proportion of human infections that may be attributed to broiler meat is unknown, there is some uncertainty as to the actual reduction in human cases that may be obtained from controlling *Campylobacter* in broiler meat. However, it has been suggested that 20–25% of the human cases acquired in Denmark can be attributed to fresh, chilled broiler meat [4].

The first Danish risk assessment from 2001 [9] included only the Danish production of broiler meat. Due to the fact that imported broiler meat also contributes to human exposure to *Campylobacter* in Denmark, the risk assessment is at the point of being updated to also include imported broiler meat.

In order to bring the number of human cases down further, novel initiatives were included in the Danish action plan from 2008. Currently, flocks are scheduled for channelling based on the *Campylobacter* status determined by the companies' own sampling and analysis of sock samples using PCR. These samples are collected 7–10 days prior to slaughter. Consequently, there is a risk that negative flocks may become colonized before slaughter and proceed to production of chilled products. The channelling approach could be optimized by using rapid detection methods with similar or increased sensitivity and specificity, allowing for sampling closer to the time of slaughter [28]. Development of new rapid detection methods was therefore included in the action plan.

In Denmark, as in other countries, a strong seasonality is observed for *Campylobacter* prevalence in broiler flocks [22, 29, 30]. This complicates flock channelling. During the high-prevalent period (June–November), fewer *Campylobacter*-negative flocks are available for chilled meat production and due to logistic difficulties it is not feasible to satisfy the consumer demand using only *Campylobacter*-negative flocks. The option of freezing all domestically produced, contaminated meat is not feasible, because this would cause a shortage of chilled products during periods, where *Campylobacter* prevalence in broilers is high. This in turn might lead to increased imports of chilled broiler meat in order to sufficiently satisfy consumer demand. Based on the historical data on *Campylobacter* in imported broiler meat, this might lead to more contaminated meat on the market, and consequently more human infections. Hence, there is a need to look into control measures other than channelling that could be applied, especially during the high prevalent period. Examples of control measures that were described in the action plan were fly screens, which have proven very effective in preventing introduction of *Campylobacter* in broiler houses under Danish conditions [31] and steam ultrasound treatment of broiler carcases [24].

Even though Denmark carries out intensive monitoring of broilers and broiler meat and has a notification system for human campylobacteriosis, it has been difficult to evaluate the impact of the implemented control strategies in general, and on human health in particular, because several factors influence the number of *Campylobacter* cases. First, broilers are not the only source of human infections, and therefore cases would occur even if *Campylobacter* was eradicated from the broiler industry. Second, several strategies are voluntary, leaving the level of compliance uncertain. Finally, national control strategies mainly affect broilers and broiler meat produced domestically, while import of broiler meat will inevitably affect the overall *Campylobacter* status of retail broiler meat within a country. This is why the new

Danish action plan also includes initiatives directed against imported broiler meat, e.g. case-by-case based surveillance [1], and the request to retailers and wholesalers to enforce stricter requirements for food safety from their suppliers.

Across Europe other countries have also implemented strategies to control *Campylobacter*, in particular countries in Northern Europe [2]. The interventions that have generally been implemented are elements of biosecurity, improved hygienic practices at slaughter and consumer education. Similar to Danish observations, countries with a strategy to control *Campylobacter* in broilers (e.g. Sweden and Norway) have seen a reduction in the prevalence of *Campylobacter* in broiler flocks and broiler meat, but not a significant drop in the number of human campylobacteriosis cases; however, there have been indications of decreasing trends [2, 22, 23]. In these countries, the actual effect might also have been counterbalanced by other factors. The only country that has clearly experienced a dramatic decrease in human cases of campylobacteriosis following implementation of control measures in broiler production is Iceland. Since strict control measures were implemented along the whole food chain (birds to humans) in 2000, campylobacteriosis cases fell from 116 cases/100 000 population to <10 cases/100 000 population [10, 32]. The control measures comprised biosecurity at farm, freezing of meat from *Campylobacter*-positive flocks and intensive consumer education campaigns. One of the major differences between Iceland and other Northern European countries is that only domestic broiler meat is consumed in Iceland. This makes national control initiatives much more effective.

In conclusion, the implemented strategy to control *Campylobacter* in Denmark has had a positive effect; reducing the prevalence in broiler flocks and broiler meat. A small decrease in the number of human cases has also been observed. The explanation for the effect on human cases not being more significant is probably due to other factors counterbalancing the effect of the implemented interventions. In particular, imported broiler meat and other sources of infection than broilers are assumed to have influenced the limited effect on humans. To combat *Campylobacter* it is important to focus not only on domestic production, but also on imported products and therefore shared activities among broiler-producing countries are essential.

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DECLARATION OF INTEREST

None.

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Manuscript II

Seasonal influence on the prevalence of thermotolerant *Campylobacter* in retail broiler meat in Denmark

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ABSTRACT

In Denmark, the incidence of human campylobacteriosis cases, as well as the *Campylobacter* prevalence in broiler flocks, is strongly influenced by season with a summer peak in July–August. Therefore, it was considered that the prevalence of *Campylobacter* in broiler meat sold at retail in Denmark might also be influenced by season. A retrospective survey analysis was performed on 2001–2007 national surveillance data of the prevalence of thermotolerant *Campylobacter* in all conventional broiler flocks at slaughter, and in randomly sampled broiler meat at retail. There was a significant effect of season on the occurrence of *Campylobacter* in meat at retail; the largest effect was found for domestic chilled meat. Thus, the *Campylobacter* prevalence in Danish broiler flocks, which fluctuated with season, was found to be a strong predictor for the occurrence of *Campylobacter* in fresh, chilled, Danish broiler meat. However, besides flock prevalence, there was also a direct effect of season on the occurrence of *Campylobacter* in Danish broiler meat at retail.

1. Introduction

Thermotolerant *Campylobacter* has been the most frequently reported gastrointestinal bacterial pathogen in Denmark since 1999 and in the EU since 2005 (Anonymous, 2009; European Food Safety Authority, 2009b). *Campylobacter* is readily isolated from poultry and poultry meat (Anonymous, 2009; European Food Safety Authority, 2009b) and it is believed that a large part of Danish food borne campylobacteriosis cases can be attributed to chilled broiler meat (Wingstrand et al., 2006).

The prevalence of *Campylobacter* in broiler flocks, as well as the incidence of campylobacteriosis in humans, has been observed to fluctuate during the year, in Denmark and in other countries such as Norway, Sweden, and the Netherlands, peaking in the warmer summer months (Anonymous, 2009; Berndtson, 1996; Jacobs-Reitsma et al., 1994; Jore et al., 2010; Kapperud et al., 1993; van Asselt et al., 2008). Large differences in the broiler flock prevalence between summer and winter have been observed; from approximately 10–60% in Denmark (2004–2007), from 50 to 100% in the Netherlands (1992–1993), and from 0 to 47% in Norway (1990–1991). Furthermore, the fluctuations in *Campylobacter* prevalence in broilers and human campylobacteriosis cases have been shown to be commensurable (Jore et al., 2010).

Broiler meat typically becomes contaminated with *Campylobacter* during slaughter (most commonly during the processes of defeathering and evisceration) or during the processes of dressing and/or trimming. The source of contamination may be the broiler itself or other *Campylobacter* contaminated carcasses or equipment (Rosenquist et al., 2006). Since the *Campylobacter* prevalence in broiler flocks, in some countries, increases during the warmer months of the year, more positive broilers are being processed during these periods, and therefore it is likely that more meat gets contaminated compared with periods with lower prevalence in broilers. Furthermore, the type of cold temperature treatment (chilling versus freezing) and the origin of the meat (domestic versus import) are also considered risk factors for *Campylobacter* contamination of broiler meat in Denmark. This is because freezing reduces *Campylobacter* levels (Bhaduri and Cottrell, 2004; Georgsson et al., 2006; Lee et al., 1998; Solow et al., 2003; Zhao et al., 2003) and because *Campylobacter* occurrence on broiler meat differs largely between countries (European Food Safety Authority, 2009a; European Food Safety Authority, 2010).

Since broiler meat is considered to be the main vehicle for human campylobacteriosis cases in Denmark, it would be appropriate to study seasonal fluctuations in the meat to support the link to the human campylobacteriosis cases. Very few European studies regarding the seasonality for *Campylobacter* occurrence in retail broiler meat have been published. In Wales, distinct seasonality was determined for chilled broiler meat at retail (Meldrum et al.,

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2006), while a study from Northern Ireland could not demonstrate seasonal fluctuations (Wilson, 2002).

The purpose of this study was to 1) investigate how the prevalence of *Campylobacter* in broiler meat (chilled and frozen), at retail, in Denmark is influenced by the origin of the meat (domestic or imported), and season, and 2) to investigate the direct effect of seasonality on the occurrence of *Campylobacter* in chilled, domestically produced broiler meat.

2. Material and methods

2.1. Sampling

During the period 2001–2007, all broiler flocks slaughtered in Denmark were tested for *Campylobacter*. Cloacal swabs were collected from 10 individual broilers per flock prior to slaughter. During the same period, samples of fresh chilled and frozen imported and Danish produced broiler meat were randomly collected nationwide from local retail establishments or wholesale outlets. The samples comprised a selection of whole carcasses and parts of chicken. According to the sampling plan the retail samples were to be collected in equal numbers every month throughout the year. The distribution of the samples collected is illustrated in Fig. 1. Regional laboratories for the Danish Veterinary and Food Administration performed sample collection and microbiological analysis.

2.2. Microbiological analysis

All analyses were done according to the methods used in the national surveillance program.

From all broiler flocks slaughtered in Denmark, 10 cloacal swabs from each flock were pooled into one sample for analysis. Detection of thermotolerant *Campylobacter* was performed by PCR as described by Lund et al. (2003). However, in 2001, a traditional cultivation method was used for detection of *Campylobacter* (Anonymous, 2007).

Methods for detection of thermotolerant *Campylobacter* in broiler meat have changed slightly over the years. In general, the detection has been based on selective enrichment followed by culture on a selective media (Anonymous, 1990; 2007). In brief, one equivalent of broiler meat (minimum 15 g) was stomached for 120 s with 8 or 9 equivalents of broth (2001–2002: 9 equivalent of Mueller-Hinton broth (MHS) (Difco, Becton Dickinson, Sparks, MD, USA); 2003–2007: 8 equivalents in Bolton broth (Oxoid, Basingstoke, UK) with supplements (sodium pyruvate 0.25 mg/l, sodium metabisulphite 0.25 mg/l, ferro sulphate 0.25 mg/l (Oxoid)), cefoperazone 30 mg/l (Sigma–Aldrich, Saint Louis, MO, USA), and trimethoprim lactate 66 mg/l (Sigma–Aldrich)). A collaborative trial demonstrated no statistically significant difference between the method used in 2001–2002 and the method used in 2003–2007 (Rosenquist et al., 2007).

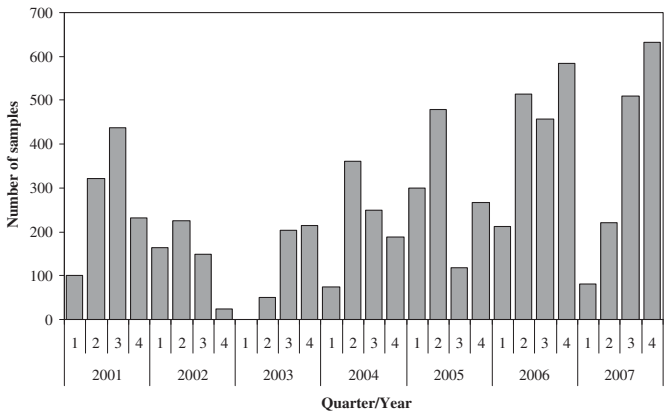

Fig. 1. Distribution of the total number of samples collected at retail from 2001 to 2007 by quarters of the year; Danish and imported, chilled and frozen meat.

For enrichment, dilutions were incubated in a microaerobic atmosphere at 41.5 ± 1.0 °C for 48 ± 4 h. Approximately 10 μl of inoculum from each dilution was streaked onto modified Cefaperazone Charcoal Deoxycholate agar (mCCDA) (Oxoid) or Abeyta Hunt Bark (AHB) agar with 0.1% triphenyl tetrazolium chloride for red-staining of colonies. The inoculated plates were incubated in a microaerobic atmosphere at 41.5 ± 1.0 °C for 48 ± 4 h. From each plate, five presumptive *Campylobacter* colonies were verified by phase-contrast microscopy, oxidase reaction, and hydrolysis of hippurate.

2.3. Data analyses

Qualitative results, categorizing if a sample was *Campylobacter* positive or negative, were obtained from the microbiological analysis. Broiler meat samples were categorized as negative if *Campylobacter* were not detected (detection limit: <0.4 cfu/g and <0.1 cfu/g for the years 2001–2002 and 2003–2007, respectively). Samples were categorized as positive whenever *Campylobacter* were detected.

Initially, univariate logistic analyses were carried out; testing the unconditional association between risk factors and *Campylobacter* occurrence. The tested risk factors were the origin of the sample (domestic, import) and the type of cold temperature treatment (chilled, frozen).

Secondly, two multivariable analyses were performed using logistic regression. The outcome variable was the occurrence of *Campylobacter* positive samples. Two-way interactions were considered during modelling. The models were developed using backwards elimination.

In the first multivariable analyses, how the occurrence of *Campylobacter* in retail broiler meat in Denmark was influenced by origin of meat (domestic or imported), type of cold temperature treatment (chilled or frozen), and season and year was investigated. Two analyses were performed; one investigating the impact of season (model 1a), and another considering the effect of years (model 1b). The risk factors, sample origin and type of chill, were combined into one variable of four levels. Seasonality was incorporated using a cosine function with a period of 12 months (Stolwijk et al., 1999). Year, specified as a classification variable (was applied only in model 1b).

In the second multivariable analysis the association between *Campylobacter* prevalence in broiler flocks, season, and year and the occurrence in meat was investigated (model 2). This analysis only included Danish broiler meat, because prevalence data were available only for Danish broiler flocks. Furthermore, broiler meat data covered only the Danish chilled meat since the longer shelf life for the frozen meat would obliterate the direct relationship between the broilers and the meat from these.

In the logistic regression analyses, the likelihood ratio test and a significance level of 5% for assessment of the significance of risk factors was used. The fit of the final models were assessed using the Hosmer & Lemeshow Goodness-of-fit. Analyses were performed using the procedure LOGISTIC in the SAS 9.1.3 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Season

Prevalences for the retail meat could consistently be grouped into two distinct periods; with low prevalences observed from December one year to May the next and high prevalences observed from June to November (data not shown). The domestic chilled

meat was the product exhibiting the largest difference between the high prevalence and low prevalence periods (Fig. 2). The distance between the curves of the observed data was wide and the confidence limits tended not to overlap.

Univariate analysis of the broiler meat data showed that the origin of the sample and the type of cold temperature treatment were individually strongly significant ($P < 0.0001$) (Table 1). The origin was the most important risk factor for *Campylobacter* on broiler meat with an OR of 3; i.e. the risk of *Campylobacter* contamination of broiler meat was three times larger for imported meat compared to domestic meat. The type of cold temperature treatment had an OR of 2.5 indicating that the chilled meat had over two times greater risk of being contaminated with *Campylobacter* compared to frozen meat.

From multivariable analysis (model 1a) it was evident that both season and the origin of the sample were significant factors ($P < 0.0001$) for predicting the occurrence of *Campylobacter* in broiler meat, but also the interaction between the two variables (season and origin of the meat) was significant ($P < 0.0001$).

When the time frame (years) was included in the analysis (model 1b), the seasonality remained a significant risk factor for all meat categories ($P < 0.05$); Danish and imported, chilled and frozen meat. Logistic analysis showed that there was no interaction between year and season ($P > 0.05$), while interactions were significant between years and origin, and season and origin. Considering each meat category individually, seasonality was significant for all four categories.

During the seven years studied, a significant decreasing trend was observed in the high prevalence periods for the imported chilled meat. In contrast, significant increasing trends were observed for the high prevalence period, for both Danish and imported frozen products. Neither increasing nor decreasing trends was evident for the other meat categories.

3.2. Impact of Campylobacter prevalence in broiler flocks on the Campylobacter occurrence on broiler meat

From the logistic analysis (model 2), the broiler flock prevalence was shown to have an impact on the *Campylobacter* occurrence in the broiler meat samples ($P < 0.0001$). However, season also had an impact on the model ($P < 0.05$). Furthermore, the variable year could not be disregarded ($P = 0.01$). No interactions were significant ($P > 0.05$). Within the range of data, the modelled relationship was linear.

The predicted mean values for occurrence in chilled Danish broiler meat coincided with the oscillating observed prevalences (Fig. 3). The predicted curve showed a slightly increasing trend over the years. The broiler flock data comprised prevalences from 10 to 80%. Consequently, the model is supported by data in this interval and it does not allow identification of the events following low and extremely high prevalence in broiler flocks.

4. Discussion

This study demonstrated the seasonality of *Campylobacter* occurrence in chilled Danish broiler meat. While similar results have been shown in studies conducted in the Netherlands and North Carolina, USA (van Asselt et al., 2008; Willis and Murray, 1997), seasonality is infrequently examined when studying *Campylobacter* occurrence in poultry meat at retail (Mena et al., 2008; Stoyanchev et al., 2007; Uyttendaele et al., 1999). Seasonality in *Campylobacter* colonization of broiler flocks is well recognised in the Nordic countries (Berndtson, 1996; Guerin et al., 2007; Jore et al., 2010; Kapperud et al., 1993; Patrick et al., 2004). Since *Campylobacter* colonized birds are a precursor for *Campylobacter* contaminated meat, this would be a reason for expecting seasonality in *Campylobacter* contamination of the fresh broiler meat as well. As a consequence of this, seasonality should be taken into account in national monitoring of *Campylobacter* occurrence in broiler meat. Where there are seasonal peaks, results of short-term observational studies may be skewed, as discussed by Meldrum et al. (2006). Additionally, where seasonality is observed, sampling during long-term studies should be done carefully as even sampling will be important to achieve credible estimates.

For the imported, chilled meat, seasonality was not found to be very distinct, but was probably masked by the interference introduced by pooling data from samples imported from different countries into the same category. Countries have different *Campylobacter* baselines (European Food Safety Authority, 2010), seasonalities, procedures for handling products, and procedures for export, etc., all of which influence the overall result.

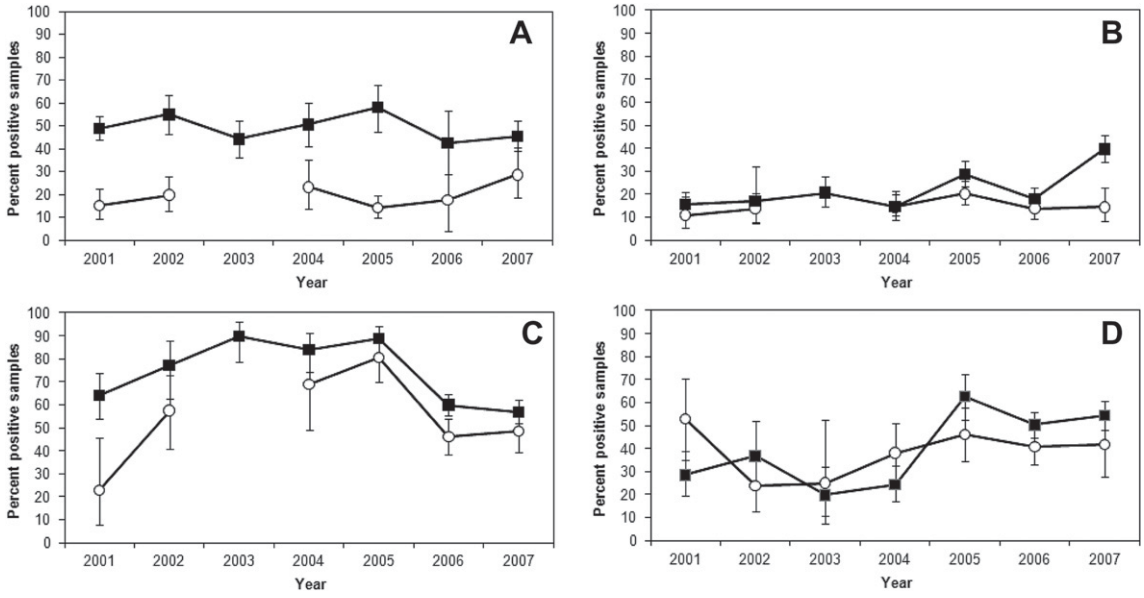

Fig. 2. Prevalence estimates calculated from the observed data stratified by sample origin, 2001–2007. Prevalence (Dec–May) (■); prevalence (Jun–Nov) (○). A) Danish chilled broiler meat, B) Danish frozen broiler meat, C) imported chilled broiler meat, D) imported frozen broiler meat. Bars indicate 95% confidence limits of the prevalence estimates.

Table 1
Parameters (estimates, odds ratios, and *P*-values) from univariate logistic analysis of the variables sample origin (Danish/import), and type of cold temperature treatment (chilled and frozen).

Variable	Level	Estimate	OR	95% Confidence limits
Sample origin	Danish	−1.087	1	
	Import	0.000	3.0	2.7–3.3
Type of cold temperature treatment	Chilled	0.896	2.5	2.2–2.7
	Frozen	0.000	1	

No noticeable seasonality was observed for *Campylobacter* occurrence in frozen meat; neither in domestic nor imported meat. Seasonal fluctuations in the occurrence of *Campylobacter* may be influenced by the longer shelf life for this type of product. This makes it difficult to relate products to a specific time period and may obliterate the impact of season. The longer shelf life for frozen meat, compared to chilled meat, can result in an extended time before the products become available to the consumer as companies can store the products and release them according to demand. Furthermore, the products will be available in the counter at retail for a longer time. Consequently, it is reasonable to expect seasonality in frozen meat at retail to be less distinct than in chilled products.

Including year as a variable in the general model (model 1b) showed no interaction between year and season. This suggests that trends for the high and low prevalence periods have been similar in the study period (2001–2007). As different trends in the high and low prevalence periods have not been observed for Danish broiler flocks this result was expected for the Danish broiler meat. With regard to the imported broiler meat, different trends were not expected because of the interference introduced by pooling different countries into the same category.

The occurrence of *Campylobacter* in chilled Danish broiler meat was found to be highly influenced by the *Campylobacter* prevalence in Danish broiler flocks. This was expected as *Campylobacter* from the broiler flock is the most likely source of *Campylobacter* in the meat. As the *Campylobacter* prevalence in broiler flocks has been observed to fluctuate with season (Anonymous, 2009), the seasonality was also expected for the meat. However, season itself also contributed to the occurrence in the meat. As no interaction between season and broiler flock prevalence was significant, the explanation for this additional effect is not linked directly to the broiler flock prevalence. It was assumed, though, that the explanation should be found in the production line before the retail level. This is, firstly, because *Campylobacter* will not multiply in meat at retail, either in summer or winter. Even if there are breaks in the chill chain, *Campylobacter* do not grow below 32 °C (Hazeleger et al., 1998). Secondly, Danish broiler meat is packaged for retail at the slaughter plant and therefore not handled directly in stores, which prevents cross contamination at this stage.

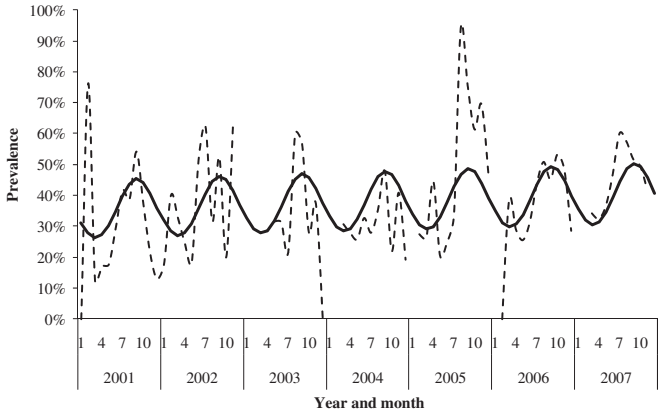

Fig. 3. *Campylobacter* prevalence in Danish chilled broiler meat; observed (- - -) and predicted values (—), 2001–2007 by month.

The additional effect of season on the *Campylobacter* occurrence in Danish chilled broiler meat is not readily explained. Several factors which differ with season may be of importance. The infection pressure of *Campylobacter* from outside the broiler house is higher in warm than in the cold periods, which leads to higher prevalence in broiler flocks (Hald et al., 2004, 2007). This also results in an earlier introduction of *Campylobacter* into the broiler house and higher within flock prevalence at the time of slaughter (Katsma et al., 2007). Furthermore, higher mean concentrations in the intestines of the broilers at the time of slaughter have been found in warmer periods (Denis et al., 2007; Manfreda et al., 2006). Higher within flock prevalences as well as higher numbers of *Campylobacter* in the intestines of broilers elevate the probability of meat contamination (Nauta et al., 2009). Finally, the explanation could be something completely different. For example, having less experienced staff (vacation temps) processing the meat during the summer holidays, or the fact that it may be easier to effectively schedule freezing all positive flocks in winter, when few flocks are positive, etc. It is important to identify the expected relationship between *Campylobacter* prevalence in broiler flocks and the meat from these, but it is also important to recognise that the broiler flock prevalence may not be the only factor influencing the *Campylobacter* occurrence on broiler meat. Pinpointing the exact risk factors may allow for more cost effective management strategies; potentially saving money if some interventions can be targeted to apply only in periods with elevated risk.

Analysing data from retail sampling also revealed that the collection of retail samples was not done evenly throughout the years. Uneven sampling within years combined with a distinct seasonality in *Campylobacter* occurrence may lead to an either too high or too low annual mean prevalence and complicates comparison of prevalences between years. The importance of even sampling is therefore emphasized with regard to quality of data, especially with regard to specific measures; e.g. baseline prevalence estimates. However, in the present study the seasonality has been accounted for and therefore data are considered commensurable.

With the results from the present study in mind, both seasonality, as well as even sampling, should be considered prior to data generation and evaluating of the *Campylobacter* situation in a given country. Additionally, chilled and frozen meat should not be pooled in the same estimates as *Campylobacter* prevalence is influenced by the chilling method. This also applies for domestic versus imported broiler meat.

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Manuscript III

Reduction of Thermotolerant *Campylobacter* Species on Broiler Carcasses following Physical Decontamination at Slaughter

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ABSTRACT

To reduce the incidences of human *Campylobacter* infections, a number of countries are investigating methods for reducing human exposure to *Campylobacter* from broiler meat. In addition to implementing biosecurity measures at the farm, *Campylobacter* may be controlled by reducing *Campylobacter* counts through physical decontamination of the meat. The current study was conducted to compare the *Campylobacter*-reducing ability of three physical decontamination techniques, forced air chilling, crust freezing, and steam-ultrasound, performed in the plant with naturally contaminated broiler chickens. The effects of all three techniques were evaluated and compared with the effect of freezing. Mean reductions obtained were 0.44 log CFU per carcass, 0.42 log CFU per sample, and ≥2.51 log CFU per carcass, respectively. All techniques resulted in significant reductions of the *Campylobacter* concentration on the carcasses ($P < 0.05$). However, none of the techniques were as effective as freezing based on reductions in *Campylobacter* counts and on adverse effects. The increase in *Campylobacter* counts on carcasses following visceral rupture during the evisceration operation also was examined. Visceral rupture resulted in an increase of 0.9 log CFU per carcass, suggesting that *Campylobacter* counts also may be reduced by optimizing the hygienic design of equipment or by physical removal of fecal contamination.

Campylobacter spp. continue to be the most common cause of human gastrointestinal disease in Europe, as in preceding years *(19).* In risk factor studies, poultry meat *(20, 34),* especially fresh and unfrozen broiler meat, has been identified as the major risk factor for campylobacteriosis *(48).* As a consequence, several countries have developed quantitative risk assessments to support management decisions regarding the control of this pathogen *(24, 29, 32, 33, 39).* These risk assessments suggest that reduction of the *Campylobacter* concentration on broiler chicken meat will impact considerably the number of human cases of campylobacteriosis.

Although good hygienic practices during rearing and processing of broilers may reduce the occurrence of *Campylobacter,* total elimination of the organisms from live poultry and poultry meat is not possible through hygienic measures alone *(2, 12, 22).* To obtain a further reduction, various decontamination techniques must be used.

Chemical decontamination of broiler carcasses has been used in the United States for several years. Some of the most commonly used antimicrobial substances are acidified sodium chlorite, chlorine, chlorine dioxide, trisodium phosphate, cetylpyridinium chloride, ozone, and peroxyacetic acid *(36, 37).* In the European Union, chemical decontamination of foods of animal origin has been allowed since Regulation (EC) No. 853/2004 *(13)* came into force in January 2006. However, no such chemicals have actually been authorized by the Commission because none of the substance applications have been approved by the European Food Safety Authority because of insufficient documentation *(14–18).*

Several physical decontamination techniques, including freezing, irradiation, and steam, have been effective against *Campylobacter.* Using these techniques, as little as a 0.5-log reduction in *Campylobacter* counts and as great as total elimination of *Campylobacter* has resulted *(9, 21, 30, 41, 43, 49).* Each method has its advantages and disadvantages with relation to appearance of the final product, consumer acceptance, price, etc. For example, although irradiation is very effective, it is expensive and will meet considerable resistance from consumers, especially in Europe *(10, 23, 47).* Therefore, this decontamination technique is not considered a viable management option. Freezing of broiler meat is also effective for obtaining marked reductions in *Campylobacter* counts, and researchers have predicted that application of this technique will significantly decrease the incidence of human campylobacteriosis *(39).* Freezing already has been implemented as an intervention in Iceland, Norway, and Denmark *(21, 25).* In Denmark, this intervention has been used on a voluntary basis where practical and possible. However, freezing of meat from all *Campylobacter*-positive broiler flocks in Denmark is not a feasible option because it would limit the marketing of domestically produced chilled broiler meat during periods when *Campylobacter* prevalence is high. Consequently, the import of chilled meat would have to increase to satisfy consumer demands. Because imported meat historically has had a higher *Campylobacter* prevalence than found in domestic product *(4),* the *Campylobacter* contamination problem in chilled chicken meat sold in Denmark would be aggravated further. Significant *Campylobacter* reduction and mainte-

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nance of product quality has been difficult to achieve with steam treatment because of the boiled appearance of the skin or meat surface *(28, 46).*

Ideally, an appropriate physical decontamination technique would be acceptable to consumers while still leaving the meat fresh with no change in product quality. To date, only forced air chilling and crust freezing meet these criteria. However, the effectiveness of these methods against *Campylobacter* has been variable between studies. A new decontamination technique, Sonosteam, is being developed based on simultaneous treatment of the meat surface with steam and ultrasound. Ultrasound enhances the killing effect of steam by efficiently removing the protective air on the meat surface. However, the effectiveness of this method against *Campylobacter* has not been investigated.

Because fecal contamination of carcasses during slaughter increases the concentration of *Campylobacter (8)* on these carcasses, reducing the incidence of fecal contamination would be a useful supplement to physical decontamination methods.

The aim of this study was to investigate the reduction of concentrations of naturally occurring thermotolerant *Campylobacter* species on broiler carcasses during industrial processing after the in-plant application of three decontamination methods: forced air chilling, crust freezing, and steam-ultrasound. The results obtained were to be used in a quantitative risk assessment of the cost-effectiveness of selected interventions during broiler production. The reductions obtained by forced air chilling, crust freezing, and steam-ultrasound were compared with reductions obtained by freezing (data from a previous study). The increase in *Campylobacter* counts on carcasses after visceral rupture during evisceration also was examined.

MATERIALS AND METHODS

Broiler flocks. The study included carcasses and breast fillets from *Campylobacter*-positive broiler flocks processed on different days at a large Danish slaughter plant. One week before slaughter, the flocks were determined to be *Campylobacter* positive by PCR analysis of sock samples *(31).* Each decontamination technique was tested in duplicate or triplicate, i.e., on two or three flocks processed on different days.

Forced air chilling. Carcasses were chilled in a forced air chiller in accordance with plant procedures. The carcasses were carried through the forced air chiller on a continuous shackle line for 3 h to obtain an outer carcass temperature of approximately 3°C. In total, 50 carcasses were collected for analysis from each of three flocks both before (controls, $n = 25$) and after ($n = 25$) treatment.

Crust freezing. Skinless breast fillets treated in a continuous CO_2 belt freezer were fed evenly into the low temperature–freezing zone (−55°C) via the loading freezer belt. Fillets were crust frozen individually and reached an outer surface temperature of approximately −1°C after treatment, just before packaging. In total, 100 breast fillets from each of three flocks were collected for analysis: two fillets per sample, 25 control samples (before treatment), and 25 treated samples.

Steam combined with ultrasound. Carcasses were treated with steam in combination with ultrasound using a recently developed Sonosteam technique (patent DK/28.03.2001/DKA200100514). The technique was applied after evisceration but before the inside-outside bird washing procedure. A proof-of-concept treatment apparatus not developed for in-line treatment was used. The equipment was located in a container outside the plant. Therefore, carcasses were sampled inside the plant, placed in sterile plastic bags, and carried outside to the container for immediate treatment. Whole carcasses were treated individually in a treatment chamber mounted with a row of specially designed nozzles that supplied steam simultaneously with the generation of ultrasound waves for outside treatment, and a rod with nozzles was used for inside treatment. Each carcass was hung on rotating shackles and treated inside for 5 s and outside for 10 s. In total, 60 carcasses from each of two flocks were collected for analysis before (controls, $n = 30$) and after ($n = 30$) treatment.

Visceral rupture during evisceration. To examine the increase in *Campylobacter* contamination of carcasses due to visceral rupture during evisceration, carcasses with ($n = 25$) and without (controls, $n = 25$) visual fecal contamination were sampled after evisceration. In total, 50 whole carcasses were collected for analysis from each of three flocks.

Sample preparation. Carcasses were prepared as described by the U.S. Food and Drug Administration *(44)* with minor modifications. Each carcass was placed in a 3,500-ml stomacher bag with a filter (Bie & Berntsen A/S, Rødovre, Denmark), and 200 ml volume of 0.1% buffered peptone water (BPW) was added, each liter of which contained 10.0 g of peptone (BD 211677, Merck, Darmstadt, Germany), 17.5 g of sodium chloride (1.06404.1000, Merck), 3.5 g of disodium hydrogen sulfate (1.06404.1000, Merck), and 1,000 ml of distilled water. The bag was then sealed and the contents were manually massaged for 2 min. The bag was then tilted to let the liquid flow to one bottom corner, which was sanitized with 70% ethanol and then cut off with sterile scissors. Holding back the carcass and the filter, the rinsate was poured into a 250-ml sterile centrifuge tube, which was kept at 4°C for a maximum of 24 h before analysis. The chilled rinsate was then centrifuged at 13,000 × *g* for 15 min, the supernatant was discarded, and the pellet was resuspended in 10 ml of 0.1% BPW.

Breast fillets were similarly prepared by surface rinsing. Two fillets were placed in a 400-ml stomacher bag with a filter (Bie & Berntsen A/S), and 50 ml of 0.1% BPW was added. The sample was treated as above, except with a 50-ml sterile centrifuge tube and 5 ml of 0.1% BPW.

Microbiological analyses. Naturally occurring thermotolerant *Campylobacter* species in the chicken rinse were enumerated in accordance with the direct plating technique described by Rosenquist et al. *(40).* Ten-fold dilutions of the chicken rinsate were made in BPW, and 0.1 ml of the dilutions was plated onto Abeyta-Hunt-Bark agar *(44)* containing 0.1% triphenyl tetrazolium chloride for red staining of colonies.

Statistical analysis. Before analysis, bacterial counts (CFU per sample) were log transformed to approximate a normal distribution of the data. Samples in which *Campylobacter* was present but below the detection limit were given a value of one-half of the detection limit. An analysis of variance was carried out using PROC GLM within the SAS Enterprise Guide statistical software, version 3.0 (SAS Institute Inc., Cary, NC). An α value of 0.05 was used as the level of significance.

TABLE 1. Campylobacter *concentrations in samples from broiler flocks before (control) and after (treated) treatment*

Treatment	*Campylobacter* concn (log CFU/sample)[a]												Mean log reduction	SEM
	Flock 1				Flock 2				Flock 3					
	Control		Treated		Control		Treated		Control		Treated			
	Mean	*n*	Mean	*n*	Mean	*n*	Mean	*n*	Mean	*n*	Mean	*n*		
Forced air chilling	4.27 A	25	4.24 A	25	4.27 A	25	3.58 B	25	4.53 A	25	3.95 C	25	0.44	0.20
Crust freezing	3.36 A	25	2.91 C	25	3.60 B	24	3.25 A	24	3.51 B	25	3.04 C	24	0.42	0.03
Steam-ultrasound	3.60 A	30	1.67[b] C	29	4.43 B	30	1.34 D	30	ND[c]		ND		2.51	0.58
Freezing[d]	2.59 A	30	1.01[b] C	30	3.24 B	30	1.95 D	30	ND		ND		1.44	0.15

[a] Within a row, means with different letters are significantly different ($P = 0.05$).
[b] Two samples treated with steam ultrasound and 14 treated by freezing had values below the detection limit. Those samples were given values of one-half of the detection limit and were included in the analysis.
[c] ND, not done.
[d] Data from Rosenquist et al. *(40).*

RESULTS

Effects of decontamination techniques. All decontamination techniques investigated resulted in significant mean reductions of the *Campylobacter* concentrations on broiler carcasses ($P < 0.05$). The mean concentrations before and after treatment and the mean reductions for each studied flock are given in Table 1. All control samples were positive for *Campylobacter,* and all flocks were positive for *Campylobacter* 1 week before slaughter. Therefore, all samples were assumed *Campylobacter* positive before treatment. Because *Campylobacter* colonization of all broilers in a flock can occur within a few days *(3, 38, 42, 45),* this assumption was considered reasonable.

Forced air chilling resulted in a significant mean reduction of 0.44 log CFU per sample. However, the reductions obtained within each flock differed significantly from 0.03 to 0.69 log CFU per carcass. Along with a significant flock × treatment interaction, these differences indicated that the effect of forced air chilling was not consistent between flocks. Comparisons of treatment groups indicated a significance difference only between flocks 2 and 3 (Table 1).

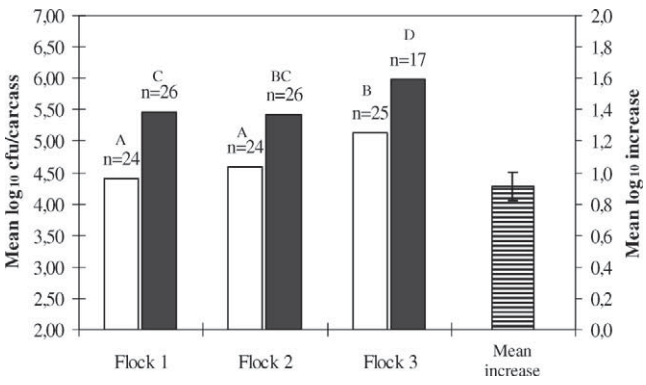

FIGURE 1. *Mean* Campylobacter *counts on broiler carcasses (log CFU per carcass) after evisceration (open columns, not visually contaminated; solid columns, visually contaminated) and mean (±SEM) count increases (hatched column). Labels indicate the number of carcasses analyzed. Letters indicate result of comparisons of means of different treatment groups (treatment and flocks) (*P *= 0.05).*

Crust freezing caused a significant mean reduction of 0.42 log CFU per sample. The standard error of the mean (SEM) for the three investigated flocks was low (Table 1), indicating that the reduction obtained by this process was consistent. This conclusion was supported by the lack of a significant interaction between the flock and treatment variables.

The steam-ultrasound treatment resulted in reductions of ≥2.51 log CFU per carcass. The exact reductions could not be determined because counts from 2 of the 30 samples from flock 1 were below the detection limit after treatment. The reductions obtained for the two flocks significantly different (Table 1), as was the flock × treatment interaction ($P < 0.05$). An adverse effect of this technique was a slightly boiled appearance of the carcass skin.

For each of the decontamination techniques investigated except forced air chilling, the initial mean *Campylobacter* concentration on the carcasses or fillets was significantly different between flocks.

Effect of visceral rupture. A higher mean concentration of *Campylobacter* (0.9 log CFU per carcass) was visually found on carcasses contaminated with fecal material than on those without such material ($P = 0.05$). Interactions between flock and treatment were not significant. The SEMs for the three flocks tested were low (Fig. 1), indicating that the increase in concentration caused by fecal contamination during evisceration was fairly uniform between flocks.

Summary. Mean reductions of 0.44, 0.42, and ≥2.51 log units were obtained by forced air chilling, crust freezing, and steam-ultrasound treatments, respectively. The steam-ultrasound method was the most effective decontamination method. Visceral rupture yielded an increase in *Campylobacter* of 0.9 log units. Therefore, as an alternative or supplement to physical decontamination, measures to reduce fecal contamination with *Campylobacter* should be considered.

DISCUSSION

All of the investigated decontamination techniques resulted in reductions of the concentration of naturally occurring *Campylobacter* species on the carcasses and fillets, with some variation in the reduction achieved. In a risk assessment, Rosenquist et al. *(39)* estimated that a 2-log reduction would result in a significant reduction in the number of human campylobacteriosis cases associated with broiler meat. Therefore, a reduction of this magnitude would be desirable for increasing the safety of *Campylobacter*-contaminated broiler chickens. A 2-log reduction in *Campylobacter* counts has been obtained by freezing the carcasses *(9, 21, 30, 43, 49).* In an earlier Danish study, the immediate mean reduction after in-plant freezing was 1.44 log units, although according to published reports the *Campylobacter* counts will decrease further with time. The decrease during storage will occur at a slower rate than that during the freezing procedure *(9, 21, 41, 43).*

Of the investigated techniques, steam combined with ultrasound applied before washing achieved the greatest reductions of *Campylobacter,* even greater than those obtained by freezing. However, a large amount of variation between replicates (flocks) was observed. Possible explanations for this could include instability of the equipment and the fact that we were not able to register the exact reduction of the first examined flock with the lowest initial *Campylobacter* concentration because the values for several of the samples after treatment were below the detection limit. The carcasses appeared to be slightly boiled after treatment. Adjustments to the equipment and treatment time were needed during the development process. Observed initial reductions of *Campylobacter* obtained by the steam-ultrasound treatment were similar to results reported previously for steam treatment (100°C) for 10, 12, and 20 s at atmospheric pressure *(28).* In that study, the carcasses also appeared to be boiled after steam treatment *(28).* Therefore, steam treatment is not a viable alternative to the steam-ultrasound technique. According to another study, steam treatments at 90°C (atmospheric pressure) for up to 24 s *(46)* caused smaller reductions of *Campylobacter* than did treatment at 100°C.

Ultrasound treatment creates an enchanced penetrating effect of steam by removing any protective surface boundary layer of air or vapor present around an object, thus allowing the steam to reach the bacteria in the microstructure and cavities on the meat surface more efficiently. This combination optimizes the steam treatment, allowing for a shorter treatment time at a high temperature. This combination has the advantage of the killing effect of steam without affecting the outer layers of the epidermis. Preliminary results with improved equipment developed for in-line industrial use have indicated minor but acceptable changes in the appearance of the poultry skin. The *Campylobacter* reductions obtained, however, have not been of the same magnitude as those in the present study, probably because the treatment time was reduced to 1 to 2 s. Although the steam-ultrasound technique has provided promising results, more studies are needed using in-line equipment to determine the actual effect on an industrial scale.

Crust freezing is a relatively new technique for rapid chilling of meat, and few microbiological studies of this technique have been published. The investigated CO_2 crust freezing technique produced consistent approximately 0.5-log reductions in *Campylobacter* concentrations. Corry et al. *(11)* found that crust freezing of chicken carcasses could be very effective for reducing *Campylobacter* counts ($\geq$2 log units), although the exact technique investigated was not specified. Various methods can be used for crust freezing, e.g., cryogenic freezing with cryogens N_2 or CO_2 or impingement freezing with cold high-velocity air. Each method could provide different results. In general, the technique is based on rapid ice crystallization on the meat surface, resulting in a thin frozen crust, followed by temperature equalization. Because *Campylobacter* is primarily a surface contaminant and is reduced by 0.5 to 2 log units during the temperature drop of ordinary freezing *(9, 21, 41, 43),* crust freezing could have additional decontamination potential. The reason for less dramatic reductions compared with ordinary freezing might be the fact that rapid freezing causes smaller ice crystals and thus less damage to the bacterial cells than occurs with ordinary freezing.

Ambiguous results have been obtained when comparing forced dry air chilling with immersion chilling for reducing *Campylobacter* counts *(1, 7, 26, 40).* However, various studies have indicated that chilling has a *Campylobacter*-reducing effect. In the present study, *Campylobacter* reductions of 0.03 to 0.69 log CFU per carcass were achieved after forced air chilling. This reduction was less than that documented in other studies: 0.8, 1.4, and 0.83 log CFU *(1, 26, 40).* The high level of variation in our data implies that the effect of this technique was not consistent between replications (flocks). The flocks were examined on different days, and the variation might be explained by adjustments to or variations in production parameters, i.e., time, temperature, and skin desiccation. Because the initial *Campylobacter* concentrations were not significantly different between flocks in the chilling experiments, initial concentration was not considered an influencing factor. If this process in general is to be relied on as an intervention step, more investigation and documentation demonstrating reliability and repeatability will be required.

The reductions obtained with crust freezing and forced air chilling were unable to meet the 2-log reduction recommendation of the Danish risk assessment *(39).* One approach to reducing the *Campylobacter* counts further would be to combine the techniques (multiple hurdle approach) to achieve synergistic or additive effects. Published studies on this subject in relation to *Campylobacter* are sparse. James et al. *(28)* combined steam or hot water with rapid cooling, chilling, or freezing and documented greater reductions when using combined treatments than when using individual treatments. However, the data were not presented in such a way as to allow evaluation of whether the effects were synergistic or additive.

In the present study, carcasses visually contaminated with fecal material after the evisceration process had sig-

nificantly higher *Campylobacter* counts than did carcasses without visual contamination. These findings are in accordance with those of Berrang et al. *(8),* who found increasing *Campylobacter* contamination on carcasses with increasing amounts of fecal material. Hygienic measures could likely reduce this contamination, for example by optimizing the hygienic design of equipment to physically remove fecal contamination (e.g., by extra washing of carcasses). The frequency of intestinal rupture differs between plants and flocks. The target in most Danish plants is a rupture frequency of less than 5%, although the actual frequency may occasionally be higher. However, an increased effort during processing will not reduce the overall level of *Campylobacter.* The reduction would apply only to the evisceration segment of the production line. Nevertheless, a reduction in this type of *Campylobacter* contamination would likely have great impact on the risk of illness, because highly contaminated meat constitutes a higher risk *(24, 29, 32, 33, 39).* Berrang et al. *(5)* suggested that it may be more efficient to intervene in the feather-removal process, where the carcasses also are contaminated with fecal material. Studies of the amount of contamination that occurs during the defeathering step all revealed increased contamination *(6, 27, 35).* However, a practical way to markedly reduce fecal contamination during processing has not yet been developed.

This study has generated information on the effectiveness of different physical decontamination techniques for reducing counts of thermotolerant *Campylobacter* strains on naturally contaminated chicken carcasses during processing. The techniques resulted in reductions of 0.4 to 2.5 log units and could be effective decontamination techniques for *Campylobacter* reduction when used alone or in combination. The steam-ultrasound method was the most effective of the three decontamination methods investigated. However, no method was as efficient as freezing when evaluating reductions in *Campylobacter* counts without adverse effects. This study also revealed the need for improved hygienic practices. However, no definitive methods were proposed.

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Manuscript IV

Effects of decontamination at varying contamination levels of *Campylobacter jejuni* on broiler meat

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Short title: Effect of decontamination at varying levels of *Campylobacter*

Key words: *Campylobacter*; Inoculation; Contamination; Decontamination; Broiler meat

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ABSTRACT

When assessing effects of decontamination techniques on counts of *Campylobacter* spp. on broiler meat, it is essential that the results reflect the variations that may exist. Decontamination studies often use high inoculation levels (10^7-10^8 cfu) and one or few strains of *Campylobacter jejuni*, and thereby restricting the results to reflect only a limited part of the 'true' situation. This study presents results from physical and chemical decontamination of broiler meat medallions using different strains and different initial concentrations of *C. jejuni.* For three strains of *C. jejuni* mean log reductions obtained by freezing at -20 °C for seven days was significantly higher and treatment with 10 % tri-sodium phosphate for 15 s was borderline significantly higher for an initial concentration of 10^7 cfu/sample on the meat compared to an initial concentration of 10^3 cfu/sample. For freezing at -20 °C for 24 h or application of 6 % tartaric acid and subsequent storage for 24 h, no statistically significant difference in reductions was found for initial concentrations ranging from 10^3 to 10^7 cfu per sample. The mean log reductions obtained by all techniques were strongly dependent on the strain tested. The results reveal that reductions obtained with high inoculation levels of *C. jejuni* (10^7 cfu/sample) and/ or single or few strains of the species should not be interpreted as a generic result for the species. If inoculation studies cannot be replaced by investigations of naturally contaminated meat, we advise to use a mixture of strains found in the production environment at levels as close to the natural contamination as possible.

INTRODUCTION

Thermotolerant *Campylobacter* species, in particular *C. jejuni*, continue to be the most common cause of human gastrointestinal disease in Europe (7). In risk factor studies, poultry meat *(9, 19)*, especially fresh, unfrozen broiler meat has been identified as the major risk factor for campylobacteriosis (26). As a consequence, several countries have developed quantitative microbiological risk assessments (QMRA) for *Campylobacter* in the broiler production chain to support management decisions regarding the control of this pathogen *(11, 14, 17, 18, 25)*. These risk assessments conclude among others that reducing the *Campylobacter* concentration on the meat will have considerable impact on the number of human cases associated with broiler meat. Consequently, decontamination of broiler meat during processing has gained increased interest as a tool to reduce the burden of *Campylobacter*.

One of the benefits of QMRAs is that the potential effects of control measures in the broiler production chain can be assessed. However, to obtain realistic risk estimates, the input data should be realistic. For example decontamination studies should preferably be carried out in industrial scale using naturally contaminated broiler meat *(5, 8)*. However, this is not always feasible, and data based on artificially inoculated samples in pilot or laboratory scale are often generated, for instance when testing newly developed equipment for decontamination or new non-approved chemical solutions.

Multiple studies on the reduction of *Campylobacter* on broiler meat by physical and chemical decontamination at slaughter using artificial inoculation have been published *(1, 3, 4, 12, 20, 21, 23)*. However, the influence of different inoculation levels on reductions is rarely included in the investigations. This issue can be of great importance, if the magnitude

of reductions is influenced by the initial *Campylobacter* concentration on the meat. In the vast majority of decontamination studies, which investigate reductions in the *Campylobacter* concentration on broiler carcasses or broiler meat, the initial inoculation levels have been high, i.e. around 10^7-10^8 cfu *(1, 3, 6, 12, 13, 15, 22, 23, 24, 27, 28)*. Hence, it is poorly understood, which reductions can be obtained for lower initial concentrations and if these are comparable to reductions obtained for high initial concentrations.

Furthermore, a great proportion of decontamination studies only consider the effect on a single strain of *C. jejuni* (*3, 4, 12, 15, 21, 23, 24*). In some cases, though, studies have included more than one strain in their evaluation, most often in a mixture (*1, 6, 13, 27, 28*), and in other cases different strains have been applied individually (*20, 22*), allowing for evaluation of strain variation. Studies of organic acid treatment and irradiation found statistical difference of reductions between strains, more or less distinct *(2, 22)*, while no strain variation was found in a study evaluating ASC and TSP *(20)*. If effects of decontamination vary significantly for different strains of *C. jejuni*, strain variation will have to be considered when planning and evaluating decontamination studies.

The aim of the present study was to investigate if the magnitude of reductions obtained by physical (freezing for 24 h and 7 days) or chemical decontamination (application of 6 % tartaric acid for 24 h or 10 % tri-sodium phosphate for 15 s) were influenced by the initial concentration of *Campylobacter* on the broiler meat and further if this reduction was strain specific.

MATERIALS AND METHODS

Experimental design. The studies were carried out in a previously developed broiler meat model (described by Birk *et al.* (2)). Three different strains of *C. jejuni,* were each inoculated onto pieces of broiler meat medallions in concentrations of 10^3, 10^4, 10^5, and 10^7 cfu/sample, and challenged by physical decontamination (represented by freezing) and chemical decontamination (represented by application of tartaric acid or tri-sodium phosphate). Each experiment was carried out in triplicate.

Preparation of broiler meat. The study included frozen skinless breast fillets from *Campylobacter* negative broiler flocks, which were provided by a Danish slaughter plant. The *Campylobacter* status was determined by PCR analysis of cloacal swabs taken upon arrival at the abattoir. In every experiment two pieces of meat were analyzed to verify the *Campylobacter* negative status of the fillets. Frozen broiler breast fillets were thawed at ambient temperature for approximately 8 hours and subsequently at 5 °C. Meat medallions of 9.6 cm^2 were aseptically cut using a plug center bit from chicken breast filets and each piece of broiler meat was placed on gauze in a petri dish.

Preparation of inocula. Three different *C. jejuni* strains were included in the study: NCTC 11168, 305, and 327. Inocula were prepared as previously described by Birk *et al.* (2) and consecutively 10-fold diluted to a final concentration of 10^4 cfu/ml.

Inoculation. For each strain, four meat medallions were prepared per decontamination technique; two for treatment and two controls (no treatment applied) (replicates). These samples were inoculated by spreading 100 µL inoculum of appropriate dilutions onto the top surface of the meat medallions with a pipette to obtain initial concentrations of 10^3, 10^4, 10^5, and 10^7 CFU/sample. To allow for diffusion of the cells, the fillet pieces were left at ambient

room temperature for 30 min. After inoculation samples were treated according to one of the following treatments.

Freezing. Samples were placed in a freezer at -20 °C for 24 hours or seven days.

Tataric acid. Samples to be treated were added 200 µL 6 % L(+)-tartaric acid (Riedel-de Haën) (25 °C) while controls were added 200 µL of sterile water (25 °C). The liquid was spread onto the surface of the meat medallions with a pipette. Samples were immediately stored aerobically at 4-5 °C with wet napkins to prevent desiccation. Sampling was carried out after 24 h.

Tri-sodium phosphate. Samples to be treated were added 200 µL 10 % tri-sodium phosphate (TSP, Sigma-Aldrich) (25 °C) while controls were added 200 µL of sterile water (25 °C). The liquid was spread onto the surface of the meat medallions with a pipette. Treatment with TSP was carried out by dipping medallions in the solution for 15 s. Sampling was carried out immediately after treatment.

Microbiological analyses. Counts of *Campylobacter* on medallions were determined by placing two arbitrary meat medallions in each stomacher bag with filter (Bie & Berntsen A/S, Denmark) and adding 10 ml of maximum recovery diluent (MRD; CM733, Oxoid). Meat medallions were stomachated for 2 min. The homogenized samples were diluted 10-fold in MRD, and 20 µl of the dilution was spotted in 5 spots onto *Campylobacter* selective AHB agar plates containing 1 % triphenyltetrazoliumchloride. All plates were incubated under microaerobic conditions for 24 to 48 h at 41.5 ± 1 °C. The *Campylobacter* colonies on the plates were counted, and CFU per sample were estimated.

Statistics. Before analysis, bacterial counts (cfu/sample) were converted to $\log_{10}$ values to approximate the data to normal distributions. Data below the detection limit were set to one

half of the detection limit. This was the case for two samples only and in each case the duplicate was countable.

Models for analysis of variance were carried out using the General Linear Models procedure within the SAS Enterprise Guide® statistical software, version 3.0 (SAS Institute Inc., USA). The significance of reduction was determined using a model including strain and initial inoculation level as classification variables and the interaction. Pairwise differences were estimated using the pdiff procedure. An α value of 0.05 was used as the level of significance.

RESULTS

Mean log reductions obtained by freezing for 24 h and application of tartaric acid were not significantly dependent on the initial concentration of the three *C. jejuni* strains inoculated onto the meat medallions (*p*-values 0.256-0.488) (Table 1). Reductions obtained by freezing for seven days, however, were significantly dependent on the initial concentration (p=0.036), i.e. a concentration of 10^7 *C. jejuni* on the meat medallions resulted in a statistically higher log reduction than a concentration of 10^3 cfu. The same was seen for TSP using the pairwise comparison, though only a borderline statistically difference was seen in the GLM analysis (p=0.064) (Table 1). The difference in reductions was only evident for the lowest (10^3 cfu/sample) and the highest level of inoculation (10^7 cfu/sample), i.e. similar reductions were obtained for inoculation levels ranging from 10^3 to 10^5 cfu. This was further documented by excluding the highest inoculum (10^7 cfu) from the analysis (Table 2).

Mean log reductions obtained by all techniques were strongly dependent on strain (*p*-values <0.001-0.031) (Table 1), i.e. significantly different reductions were obtained for the three *C. jejuni* strains included in the study; NCTC11168, 305 and 327. None of the strains were more sensitive to treatment than the others.

DISCUSSION

Several published decontamination studies on methods to reduce counts of *Campylobacter* on broiler meat, both chemical *(13, 23, 27)* and physical *(1, 3, 6, 12, 15, 22, 24, 28)* have used artificial inoculation. In many of the studies, high levels (10^7-10^8 cfu) of either a single strain of *Campylobacter* or a mixture of a few strains have been the starting point before treatment *(1, 3, 6, 12, 13, 15, 22, 23, 24, 27, 28)*. To our knowledge, only one study has looked into different inoculation/contamination levels (10). Therefore, the influence of inoculation level on the magnitude of reduction of *Campylobacter* on meat has not been well documented. When assessing the impact of a given control measure, for example in quantitative risk assessments, a realistic level of reduction is crucial to obtain credible risk estimates (16).

In the present study, statistical analysis revealed that log reductions obtained by freezing for seven days and treatment with TSP were significantly (TSP borderline) dependent on the initial concentration of three strains of *C. jejuni* inoculated onto meat medallions. A high initial concentration of *C. jejuni* (10^7 cfu/sample) gave rise to a higher log reduction than a low initial concentration (10^3 cfu/sample). This was, however, not observed for freezing for 24 h or treatment with tartaric acid. Hence, for these latter treatments, it could be reasonable

to conclude that equal log reductions are obtained for different initial contamination levels on the meat. Nevertheless, this study also revealed that reductions obtained with inoculation levels of 10^7-10^8 cfu should be interpreted with caution as they in some cases may lead to a higher effect of decontamination than seen for lower levels of *C. jejuni*. That the initial inoculation level of *C. jejuni* influenced the bactericidal efficacy was also concluded in the other reported study using different inoculation levels on beef and treatment with organic acids (10).

In the present study, reductions obtained by freezing and treatment with tartaric acid and TSP varied significantly for the three strains tested. Significant strain variation in treatment of broiler meat with organic acids have also been reported in other studies (2, 10). This means that reductions obtained with one or few strains of *C. jejuni* should not be interpreted as a general result for the species.

Our results support the statements in the EFSA guidance document on the submission of data for the evaluation of the safety and efficacy of substances for the removal of microbial surface contamination of foods (5) and the criteria in the FAO/WHO report on benefits and risks of the use of chlorine-containing disinfectants in food production and food processing (8) which state that to provide the highest body of evidence of data to be used in efficacy evaluations and risk assessments, decontamination studies should preferably be carried out in industrial scale using naturally contaminated meat. Implicit in this are natural contamination levels and more strains of *Campylobacter*. If for some reason, investigations of naturally contaminated meat cannot replace inoculation studies, we advise to use a mixture of strains found in the production environment at levels as close to the natural contamination as possible.

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TABLES

Table 1. Mean log reductions (log_{10}), standard error of the mean (SEM) and corresponding *p*-values from GLM including all inoculation levels

		NCTC11168		305		327		*p*-value		
Treatment	Conc.	mean	SEM	Mean	SEM	mean	SEM	Strain	Level	Pdiff
Freezing	10^7	-1.09	0.05	-1.08	0.02	-1.19	0.12	**0.018**	0.256	A
24 h	10^5	-0.85	0.07	-1.17	0.06	-1.13	0.04			A
	10^4	-0.83	0.08	-1.24	0.04	-1.00	0.04			A
	10^3	-0.88	0.05	-1.06	0.03	-0.91	0.04			A
Freezing	10^7	-1.49	0.20	-1.77	0.03	-1.23	0.25	**0.001**	**0.036**	A
7 d	10^5	-1.00	0.14	-1.51	0.04	-1.25	0.10			AB
	10^4	-1.36	0.12	-1.61	0.15	-1.12	0.12			AB
	10^3	-0.96	0.25	-1.43	0.14	-1.02	0.08			B
TSP	10^7	-1.23	0.09	-0.68	0.04	-0.51	0.04	<**0.001**	0.064	A
15 s	10^5	-0.79	0.08	-0.61	0.05	-0.38	0.13			AB
	10^4	-0.96	0.07	-0.82	0.08	-0.40	0.06			A
	10^3	-0.75	0.05	-0.73	0.06	-0.20	0.03			B
Tataric	10^7	-1.08	0.10	-0.90	0.03	-1.13	0.13	**0.031**	0.488	A
acid	10^5	-1.16	0.02	-0.94	0.50	-1.19	0.10			A
24 h	10^4	-1.14	0.08	-0.43	0.10	-1.11	0.12			A
	10^3	-0.88	0.14	-0.58	0.21	-1.07	0.10			A

Effect of interaction between strain and level was tested for all treatments but was not statistically significant; Statistically significant *p*-values are printed in bold

Table 2. *P*-values based on GLM including the inoculation levels 10^3, 10^4, 10^5

Treatment	Time	Strain	Level
Freezing	24 h	**0.002**	0.355
	7 d	**0.002**	0.156
TSP	15 s	**<0.001**	0.222
Tataric acid	24 h	**0.039**	0.936

Effect of interaction between strain and level was tested for all treatments but was not statistically significant; Statistically significant *p*-values are printed in bold

Manuscript V

Human risk from thermotolerant *Campylobacter* on broiler meat in Denmark

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Abstract

This paper describes a new approach by which changes over time in the relative risk of human campylobacteriosis from broiler meat are evaluated through quantitative microbiological risk assessment modelling. Danish surveillance data collected at retail from 2001 to 2010 on numbers of thermotolerant *Campylobacter* spp. on Danish produced and imported chilled and frozen broiler meat were the basis for the investigation. The aim was to explore if the risk from the different meat categories had changed over time as a consequence of implemented intervention strategies. The results showed a slight decrease from 2005 to 2008 in the human risk from Danish produced broiler meat , and a decrease from 2005 to 2010 in the risk from imported chilled meat. This risk reduction coincides with control measures implemented to reduce *Campylobacter* in Danish and imported chilled broiler meat. The human risk of campylobacteriosis from Danish frozen meat increased but remained lower compared to chilled meat. In total, the relative risk from broiler meat available for sale in Denmark increased from 2001 to 2005 after which the risk decreased to a level similar to the period 2001-2002. The use of QMRA in the evaluation of intervention strategies based on monitoring data provided an added value, compared to the traditional approach of only using changes in prevalence. The estimated human health risk is a function of prevalence and the distribution of concentrations, and therefore takes best usage of the available data, while providing the most relevant outcome for food safety risk managers.

INTRODUCTION

Thermotolerant *Campylobacter* is the most frequently reported gastrointestinal bacterial pathogen in Denmark as well as in EU *(6, 14)*. Chilled broiler meat is considered to be the largest single source of human *Campylobacter* cases in Denmark *(28)*. Monitoring of several different retail products in Denmark has furthermore shown that thermotolerant *Campylobacter* is rarely detected on fruits and vegetables *(1, 3)*, pork (minced meat) and beef (minced meat) *(2)* but more frequently in turkey meat and broiler meat in particular *(8)*. Similar observations have been reported across Europe *(15)*. In Denmark, the occurrence of thermotolerant *Campylobacter* in broiler meat has been monitored at retail since 1995.

In 2003, an action plan against *Campylobacter* in broilers was adopted in Denmark. The action plan focused primarily on improvement of biosecurity in the primary production, scheduling of *Campylobacter* positive broiler flocks to frozen production (where practical and possible), reduction of the *Campylobacter* concentration on broiler meat at slaughterhouses by freezing, and reduction of cross-contamination in domestic kitchens through consumer campaigns *(3)*. In 2008, a new four year action plan was initiated with the aim to further decrease the prevalence and the concentration of *Campylobacter* in broilers and broiler meat. The key initiatives included initiatives at all levels of the production chain. At farm level, focus was directed towards development and implementation of an industry code of practice for farmers to increase attention to biosecurity measures and development of fly screens for broiler houses, which have proven very effective in preventing introduction of *Campylobacter* in the broiler houses under Danish conditions *(17)*. Attention was also given to optimization of ante-mortem sampling to improve the scheduling of flocks. Investigation of applicable methods for decontamination and improved hygiene were the key initiatives at abattoir level. Initiatives directed towards consumers included launching of consumer information campaigns and development of educational material for

school children to improve awareness on kitchen hygiene *(3)*. Development and intensified "case-by -case control" was also part of the new action plan. This "case-by-case control" was implemented in Denmark in 2007 and aims at reducing high-risk batches of fresh meat entering the market. In practice, a number of imported and Danish batches of fresh meat are examined for the presence of *Salmonella* and numbers of *Campylobacter* and based on these results the relative risk from a batch is assessed using risk modelling. If a batch is considered injurious according to article 14 in the EU food law (Regulation (EC) 178/2002), the food producing establishments cannot market the batch and already marketed batches must be withdrawn *(8)*.

As mentioned above different initiatives have been implemented in the broiler production chain in Denmark, but the effect of measures has not been evaluated. Observing changes in the number of registered human *Campylobacter* cases will probably not answer this question clearly, as several reservoirs harbour *Campylobacter* and pose risk to humans via different pathways. Accordingly, broiler meat is considered the largest single source of domestically acquired campylobacteriosis; however, it is only one source of many. The exact proportion of cases attributable to this source/pathway combination as well as the temporal epidemiology is not known. Instead of evaluating the registered number of human cases, the *Campylobacter* status is traditionally assessed based on prevalence in for example broiler flocks and broiler meat. However, the number of bacteria ingested is believed to be of great importance in relation to human illness *(21)*. Using risk assessment models to evaluate changes in relative human risk from different meat categories could be a way to evaluate effect of action plans.

Several risk assessments on *Campylobacter* in broiler meat have been developed within the last ten years. The risk assessments are used for different purposes; i.e. to evaluate potential effects of control measures in the broiler production chain and to assess the human risk due to *Campylobacter* in broiler meat *(21)*. In Denmark, risk assessment models are also used on a day to day basis in the case-

by-case control (see above) *(8)*.

In this study, we use quantitative microbiological risk assessment (QMRA) modelling as a novel tool to evaluate changes over time in the relative risk of human campylobacteriosis from broiler meat. The data evaluated are surveillance data on numbers of thermotolerant *Campylobacter* spp. on Danish produced and imported chilled and frozen retail broiler meat from the period 2001-2010.

MATERIALS AND METHODS

Sampling. In the period 2001-2010, samples of fresh chilled and frozen imported and Danish produced broiler meat were randomly collected nationwide from local retail establishments. The samples were collected and analysed by the Danish Regional Veterinary and Food Authorities. All samples were collected at random with no knowledge of *Campylobacter* status. The number of samples collected for each category within years is presented in Table 1.

Microbiological analysis. The number of thermotolerant *Campylobacter* spp. in the samples was determined semi-quantitatively according to NMKL method 119. Revisions of the method was applied when published *(4, 5, 7)*. In brief, one equivalent of broiler meat (minimum 15 g) was stomached for 120 s with 8 or 9 equivalents of broth (2001-2002: 9 equivalent of Mueller-Hinton broth (MHS) (Difco, Becton Dickinson, Sparks, MD, USA); 2003-2007: 8 equivalents in Bolton broth (Oxoid, Basingstoke, UK) with supplements (sodium pyrovate 0.25 mg/l, sodium metabisulphite 0.25 mg/l, ferro sulphate 0.25 mg/l (Oxoid)), cefoperazone 30 mg/l (Sigma-Aldrich, Saint Louis, MO, USA), and trimethoprim lactate 66 mg/l (Sigma-Aldrich)). A collaborative trial demonstrated no statistically significant difference between the method used in 2001-2002 and the method used in 2003-2007 (Rosenquist *et al*., 2007).

As a result of this semi-quantitative method, the numbers of samples were obtained in the classes < 0.1 cfu/g, 0.1 – 1 cfu/g; 1-10 cfu/g, 10-100 cfu/g, 100-1000 cfu/g, and >1000 cfu/g for each meat category, year and season. The observed prevalence (p_{obs}) is the percentage of samples that is not falling within the first class, < 0.1 cfu/g.

The amount of meat available for sale. The amount of broiler meat available for sale in Denmark (Table 1) was obtained from the Danish Agriculture and Food Council and Statistics Denmark.

Data analyses. The *Campylobacter* prevalence for broiler meat is influenced by season *(10)*. Therefore, the annual mean prevalence for retail samples were calculated as means of the quarterly prevalences to account for seasonality. To evaluate whether concentrations were influenced by season, the difference between quarterly concentrations (in logs) was tested using an unpaired t-test. An α-value of 0.05 was considered to be statistically significant in the statistical analyses. Analyses were performed using the GraphPad QuickCalc software, Inc.

The semi-quantitative method used provides interval data and has a limit of detection (LoD) of 0.1 cfu/g. As food products may be contaminated below this LoD, the observed prevalence will most likely not be the "true" prevalence of contaminated products. To facilitate the risk assessment, a lognormal distribution was fitted to the semi-quantitative data. The estimated prevalence (p_{est}) indicates the proportion of food products for which the 10 based log of the concentrations (log cfu/g) can be described by a normal distribution with mean (μ) and standard deviation (σ). Other food products are not contaminated. Estimates for p_{est}, μ and σ were obtained for each meat category and year using maximum likelihood estimation (MLE). This allows the incorporation of censored data *(19)*.

By introducing the estimated prevalence as one of the terms in the likelihood equation, a prevalence estimate is also produced with the MLE method. The estimated prevalence p_{est} will be higher than the observed prevalence (p_{obs}) since a higher number of samples are considered to be positive due to the addition of a part of the results below the LoD. A similar method has previously been used in the EFSA Scientific Opinion on *Campylobacter* in broiler meat production *(13)*. The MLE method was performed with the Solver Add-in for Excel 2010.

Evaluation of the relative risk in total from broiler meat available for sale in Denmark could not

be done directly from the MLE fitted data as the model was not converging for some of the data. Therefore, the estimation of the total risk was based on the observed data.

The QMRA model used, combines a consumer phase model (based on M. Nauta, M. Saana, and A. Havelaar, submitted for publication) and a dose response model *(27)* as described by Nauta and Christensen *(20)* and M. Nauta, M. Saana, and A. Havelaar, submitted for publication, and has also been applied by EFSA *(13)*. The output of the risk assessment model is the mean probability of illness from consumption of a random sample of broiler meat. Relative risks of different chicken categories are calculated using Danish chilled meat in 2007 as baseline with relative risk 1. The year 2007 was chosen as baseline as this was the year before the implementation of the second Danish action plan (2008-2012).

RESULTS

***Campylobacter* occurrence.** Generally, the observed *Campylobacter* prevalence was higher in chilled meat compared to frozen meat for both Danish produced and imported meat (FIG 1). The *Campylobacter* prevalence for Danish chilled meat remained fairly unchanged within the study period whereas the prevalence for the frozen meat was increasing. For the imported meat, the prevalence decreased for both chilled and frozen meat from 2005.

The concentrations of *Campylobacter* on the chilled meat were noticeably higher than on the frozen meat for both Danish produced and imported meat. No seasonality was observed for concentrations in the four different quarters ($P > 0.05$). For the semi-quantitative data, log-normal distributions were fitted using MLE to the extent possible. The available data did not converge for all quarters; hindering calculation of risk estimates for the implicated product/year combination. From the MLE an estimated prevalence, mean concentration and standard deviation of concentrations were obtained. Figure 2 illustrates the fluctuation of mean *Campylobacter* concentration including the standard deviation of concentrations (σ). These estimates were used as input data in the risk assessment model.

Human risk. The output of the risk assessment was relative risk estimates for Danish produced and imported broiler meat. Three main outputs were generated; 1) the relative risk from each product category; for direct comparison of the risk from a random sample of Danish produced broiler meat or imported meat, (FIG 3), 2) the relative risk stratified by the proportion of broiler meat available for sale, for comparison of the overall risk from Danish produced broiler meat and imported meat (FIG 4), and 3) the relative risk in total from broiler meat available for sale in Denmark (FIG 5). From figure 3, it is seen that the risk from imported meat (chilled and frozen) in the major part of the study period was higher

compared to Danish produced meat. Figure 4 illustrates how the overall risk from Danish produced meat increases, resulting in very similar risk estimates for Danish and imported meat, as a consequence of including the proportion of meat available for the consumer. As seen from figure 3 and 4, the risk fluctuated in the study period for all the meat categories. Note the decrease in risk from imported chilled meat since 2005 and the decrease 2005-2008 followed by an increase in risk 2008-2010 from Danish produced chilled meat. The risk from Danish produced meat surpassed the risk from imported meat between 2008 and 2009. Furthermore, the risk from Danish frozen meat increased during the study period while the risk from the imported frozen meat remained fairly steady. The risk from frozen meat, however, was lower than the risk from chilled meat.

The relative risk in total from broiler meat available for sale in Denmark showed a tendency to increase from 2001 to 2005 after which it decreased until 2009 (FIG 5). The peak in 2005 was due to remarkably high mean concentrations for chilled meat, which is not readily explained. The high concentrations in Danish chilled meat in 2005 were confirmed by data from another Danish surveillance program sampling chilled broiler meat at slaughter houses (data not shown).

Figure 6 illustrates the relationship between human risk and observed prevalence. From this figure it is evident that the observed prevalence was associated with human risk; however, a strong correlation was not evident. QMRA clearly provided another risk ranking than the observed prevalence.

DISCUSSION

In this study, QMRA modelling was introduced as a tool to evaluate changes over time in the risk of human disease from a specific food source. The risk estimates are based on information on prevalence and concentration on the meat included in the evaluation.

The distributions of concentrations and in particular the high value tail are important in relation to human risk from *Campylobacter*, as high concentrations lead to high probability of illness *(21, 25)*. The tails are determined by the standard deviations of the concentrations, which are obtained from fitting lognormal distributions using a maximum likelihood estimation (MLE) approach for censored data, that can be applied to the available semi-quantitative data *(11)*. High standard deviations might reflect a 'true' large variation of the concentrations, but could also be a consequence of imprecise estimates obtained from the MLE when data were insufficient.

The available surveillance data was used as a representative base for fitting a distribution to infer the variation for the whole population (all meat available for sale). Here we chose to use the MLE for estimation of p_{est}, μ and σ by fitting a zero-inflated lognormal distribution. Zero-inflation is needed because many batches of broiler meat are not found to be contaminated. The lognormal distribution is frequently used to describe the variation of concentrations of foodborne pathogens *(11, 12, 18)*. An alternative approach would have been to apply the data sets as empirical distributions; however, as the data available were semi-quantitative, and the QMRA model requires quantitative data, we needed to make assumptions about their distribution anyway.

It would be straightforward if evaluation of the effectiveness of intervention measures of specific food sources could be measured directly on the registered number of human cases. However, broiler

meat only represents one of many sources for human campylobacteriosis, and as the specific proportion of cases attributable to broiler meat is unknown, the number of human cases is an imprecise measure for evaluating intervention effectiveness. For example, the registered number of human cases in Denmark (data not shown) did not increase coincidently with the peak in the relative risk in total from broiler meat available for sale in Denmark in 2005.

We chose to use QMRA for the evaluation of the effect of the implemented action plans, using the estimate of relative risk as a comparative measure as opposed to only considering changes in prevalence. Prevalences are frequently used to evaluate changes in contamination status of food products, however, they need not be a good indicator of the human health risk associated with the food considered *(22, 23)*, as confirmed in this study . The QMRA applied here provided a human health risk estimate based on both the prevalence and the variation in concentrations of the positive samples. Hence, our method takes full advantage of the available data. Still, assumptions in the QMRA affect the outcome of a model, e.g. concerning biological factors, consumer behaviour and the dose-response relationship.

The *Campylobacter* prevalence for Danish chilled meat sampled at retail remained fairly unchanged within the study period, 2001-2010, with a slight decrease from 2005 to 2008. This is not in complete agreement with results from another Danish surveillance program, where the proportion of *Campylobacter* positive samples of chilled meat at the large Danish slaughterhouses decreased within the period 2004-2006 *(9, 24)*. According to the sampling scheme, retail samples should represent the products available for sale. This sampling might lead to an overrepresentation of meat from the smaller producers when compared to the actual sale as the display of the smaller producers may surpass the 2% of the actual yearly sale of these products. Meat from the smaller producers often consists of special

productions, i.e. meat from older birds with a higher probability of being positive for *Campylobacter*, organic production, etc. Furthermore, the smaller slaughterhouses do not have the possibility of implementing for example scheduling. Being aware of the prevalence being higher from these productions (data not shown) the modelled risk from Danish meat might be somewhat higher than the actual risk. However, the way of sampling was consistent over years, so a bias would be consistent.

The model predicts that the risk of human campylobacteriosis from a random sample of Danish produced broiler meat decreased from 2005 to 2008 and increased in the period 2008-2010.

The slight decrease in human risk and prevalence for Danish chilled broiler meat from 2005-2008 coincides with an increase for Danish frozen meat. This might be an effect of scheduling of meat from negative flocks to chilled production and positive broiler flocks to frozen production to the extent possible, which was implemented in 2003. The increasing human risk from 2008 to 2010 was driven by increasing concentrations. Increasing concentrations in this period were observed for retail samples and samples collected at slaughter houses (data not shown).

The risk from imported chilled broiler meat decreased steadily from 2005 to 2010. A corresponding decrease was recorded in prevalence for the meat from 2005 to 2008, but not from 2008 to 2010, where it was slightly increasing. Here, the decreasing risk was driven by decreasing concentrations. The case-by-case control was put into action in late 2007 *(8)*. It is likely that this initiative has been a large contributor in reducing risk from imported meat based on increased focus on *Campylobacter* from retailers, with the incentive of not wanting to withdraw products from the market.

Human risk from frozen meat was continuously lower compared to chilled meat, even though the prevalence was not. The lower risks are due to the lower concentrations found on frozen meat, which agree with the finding that freezing reduces the *Campylobacter* concentration *(16, 26)*. The increasing

risk from Danish frozen products seems to be a consequence of scheduling of meat from positive flocks to frozen production.

The risk from a random sample of Danish produced and imported meat differed. However, the inclusion of the amount of meat available for sale increased the overall risk from Danish meat. This was a direct result of the fact that the proportion of Danish meat available for sale was higher compared with import. Though, the proportion was diminished over years.

Changes in the risk estimates were observed within the different categories of meat. Overall, an increasing tendency in the relative risk in total from broiler meat available for sale in Denmark was turned around after 2005 and kept on a stationary level with minor fluctuations. It would be attractive to study whether the observed trends in the relative risks are significant. However, this would require an uncertainty analysis for the risk estimates, and at present no method is available to us to perform this type of analysis. Furthermore, it should be stressed that this kind of study, which is based on register data, does not allow a comparison with a situation without interventions.

The present evaluation shows that the Danish initiatives in the first action plan (2003-2007) against *Campylobacter* seem to have had an effect. The scheduling directed meat from positive flocks to production of lower risk products. The case-by-case risk assessments provided an incentive for retailers to heighten standards for their suppliers. For live broilers the prevalence was reduced by intensified biosecurity *(24)*. Half way through the second action plan (2008-2010), no additional risk reductions were observed for Danish meat. Efficacy of consumer campaigns was not evaluated; it has previously been concluded that the effect of consumer information in terms of risk reduction will usually be very small *(22)*.

A national control strategy only affects the proportion of meat that originates from the domestic

production. Therefore, the effect of interventions will not target all broiler meat available for consumption and accordingly, the direct impact on human cases will be less distinct than if all meat was domestically produced. The case-by-case initiative targeting also imported meat was therefore essential in trying to cover all meat available for consumption in Denmark. It should be kept in mind that the action plans initiated in the Danish broiler production are on a voluntary basis so consistency in complying with interventions cannot be strictly assumed.

CONCLUSION

In conclusion, QMRA modelling seemed to be a valuable tool for evaluating changes over time in risk of human campylobacteriosis from broiler meat and thereby evaluating effects of action plans in this production. The QMRA performs a more detailed evaluation of the *Campylobacter* status in relation to human risk as oppose to prevalence alone. Especially for an organism as *Campylobacter*, where the number of cells is believed to be important in relation to human illness it would be an advantage to apply this kind of tool when circumstances allow for it.

The most important intervention adopted for Danish produced broiler meat seemed to be the scheduling of meat from *Campylobacter* positive broiler flocks to freezing to the extent possible. Even though the risk from frozen broiler meat has been increasing it still remained lower than the risk from chilled meat. With regard to imported meat, the case-by-case risk assessments are believed to have influenced the decreasing human risk from this meat category. In general, a tendency for an increasing risk from broiler meat available for sale in Denmark seems to be turned after 2005. Changes occurred within the different categories of meat, but as they are counterbalancing each other the total risk was not markedly decreasing in the last part of the study period when compared to the initial part of the study.

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Figure legends

FIG 1. Observed *Campylobacter* prevalence (mean of quarters) for broiler meat at retail, 2001-2010; Danish chilled meat (grey), Danish frozen meat (grey dotted), imported chilled meat (black), imported frozen meat (black dotted); ± 95 % confidence intervals.

FIG 2. MLE fitted estimates of mean concentrations of *Campylobacter* at retail (log cfu/g) for contaminated broiler meat. Error bars indicate the standard deviation of concentrations (the variability, not the uncertainty of the mean). Danish chilled meat (A), Danish frozen meat (B), imported chilled meat (C) and imported frozen meat (D), 2001-2010.

FIG 3. Evaluation of the risk from a random sample of broiler meat by direct comparison; Danish chilled meat (grey), Danish frozen meat (grey dotted), imported chilled meat (black), imported frozen meat (black dotted). Relative risk estimates of Danish produced and imported meat in Denmark, 2001-2010. Danish produced chilled meat in 2007 was the baseline.

FIG 4. Evaluation of the total risk from broiler meat in relation to the amounts available for sale; Danish chilled meat (grey), Danish frozen meat (grey dotted), imported chilled meat (black), imported frozen meat (black dotted). Relative risk estimates corrected for the amount of broiler meat available for sale of Danish produced and imported meat in Denmark, 2001-2010. Danish produced chilled meat in 2007 was the baseline.

FIG 5. Total risk based on calculations using raw data for the four meat categories; Danish chilled meat, Danish frozen meat, imported chilled meat and imported frozen meat. Risk are calculated in relative to the total risk in 2007.

FIG 6. Association between risk estimates and the observed prevalence. Each symbol represents the estimated risk corresponding to the observed prevalence for all categories for all years; Danish chill (■), Danish frozen (●), imported chill (▲), imported frozen (×).

Tables

Table 1. Number of samples taken within the categories and the proportion (prop) of broiler meat available for sale in Denmark, 2001-2010.

	Domestic chilled		Domestic frozen		Import chilled		Import frozen	
Year	N	prop	N	prop	N	prop	N	prop
2001	515	24%	336	56%	111	8%	127	12%
2002	247	24%	149	58%	79	5%	88	13%
2003	160	23%	187	54%	60	4%	80	19%
2004	178	21%	392	50%	93	7%	135	22%
2005	340	22%	547	43%	175	10%	180	25%
2006	62	25%	535	39%	662	15%	448	21%
2007	302	24%	365	41%	456	17%	264	18%
2008	757	23%	299	35%	602	16%	220	26%
2009	702	26%	548	31%	712	14%	509	29%
2010	767	26%	477	26%	580	16%	168	32%

Figures

Figure 1

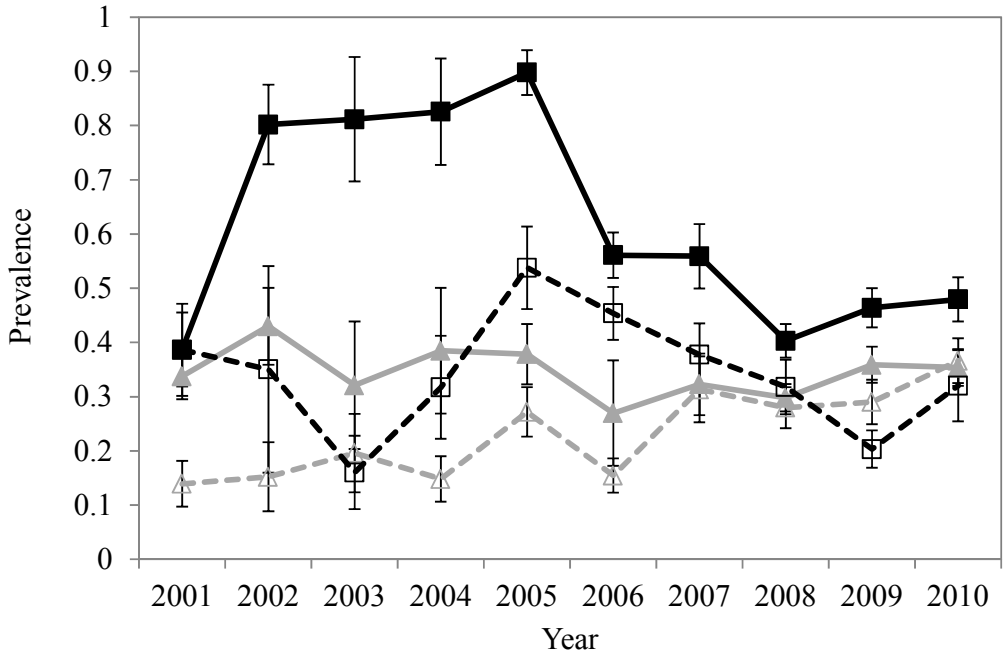


Figure 2

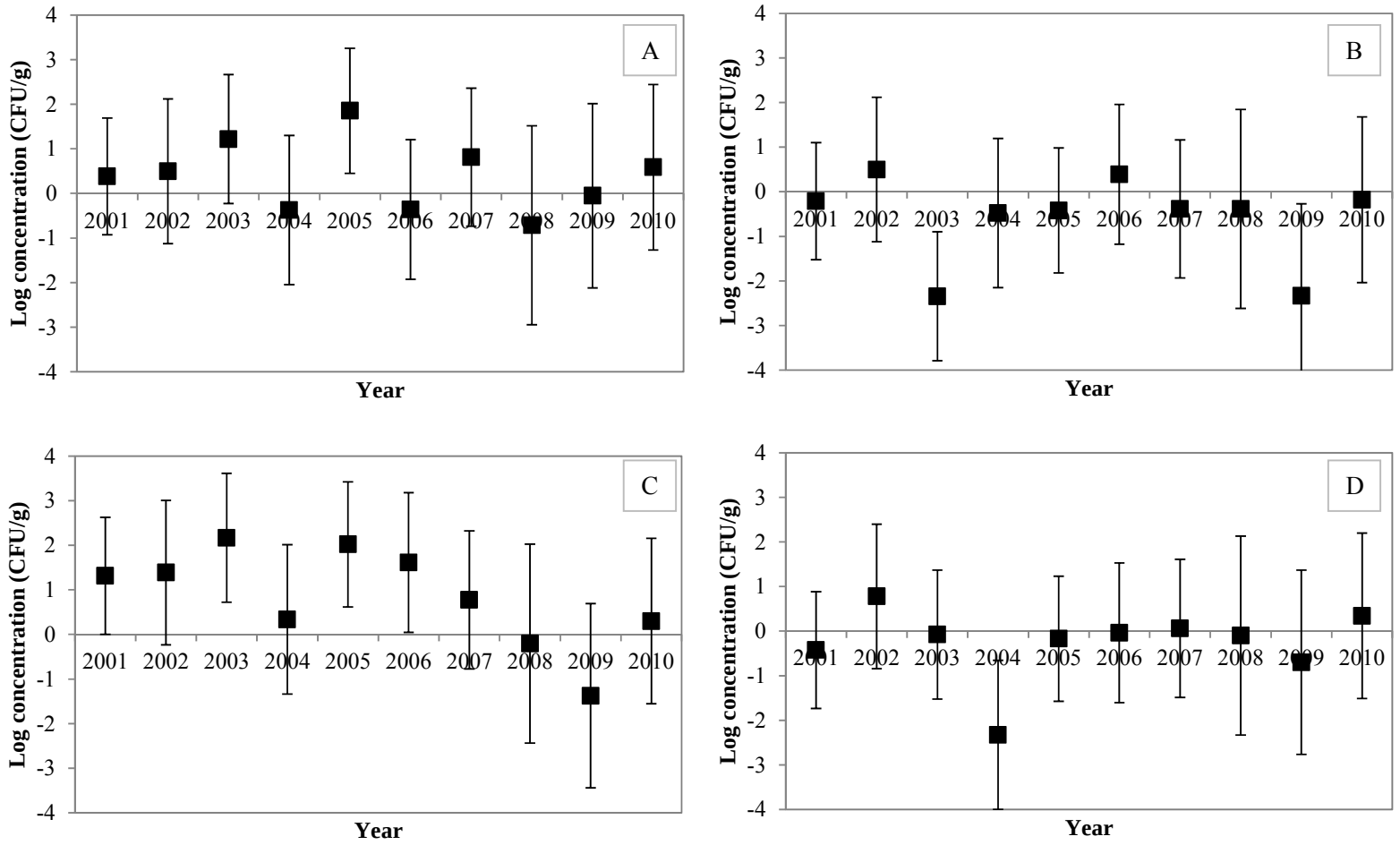

A
B
C
D
Log concentration (CFU/g)
Year
4
3
2
1
0
-1
-2
-3
-4
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010

Figure 3

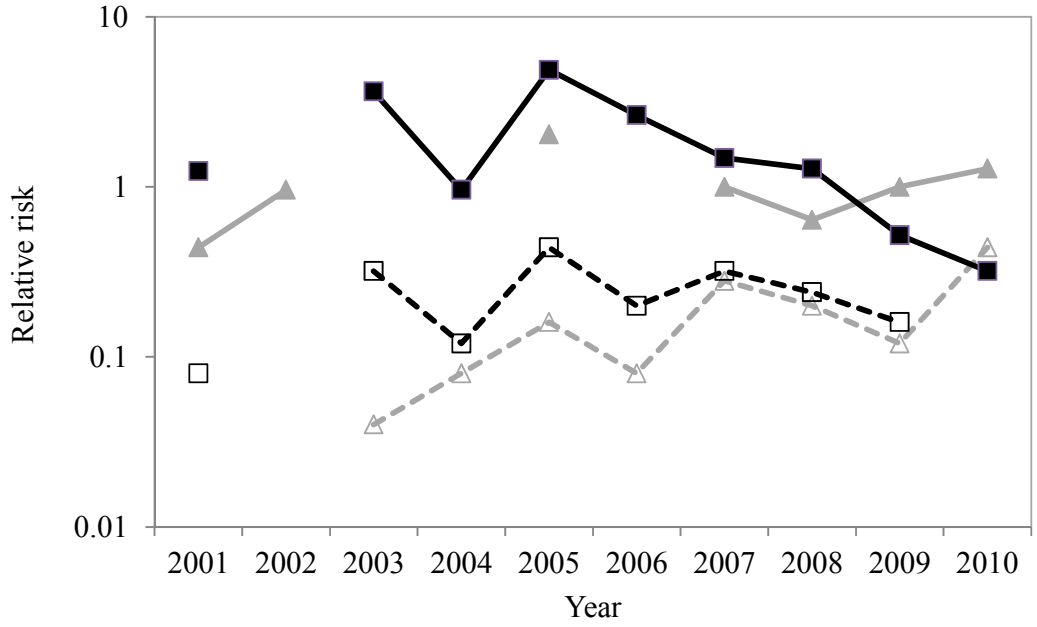

10
1
0.1
0.01
Relative risk
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
Year

Figure 4

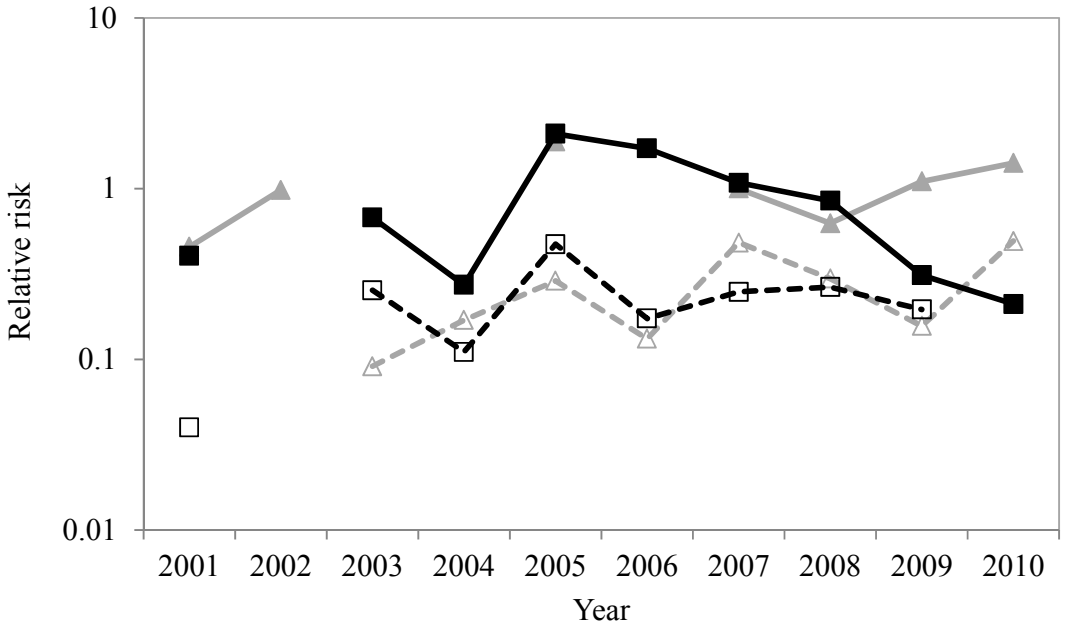


Figure 5

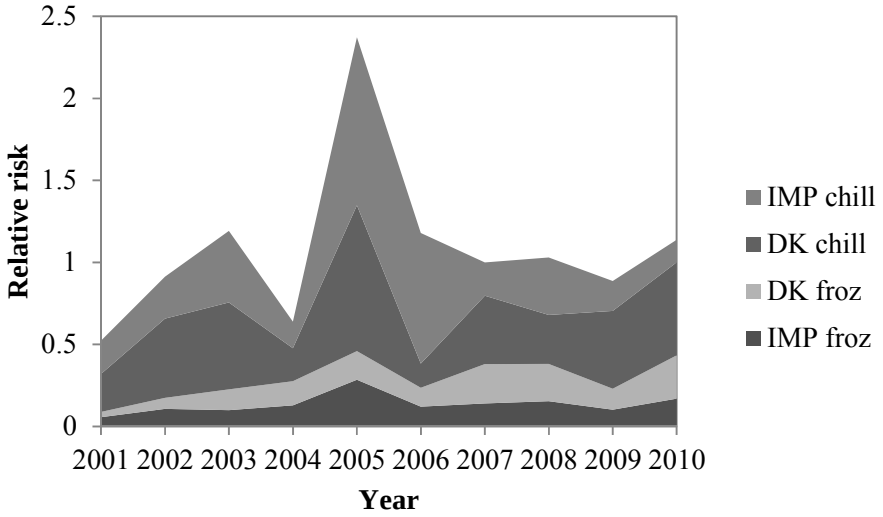
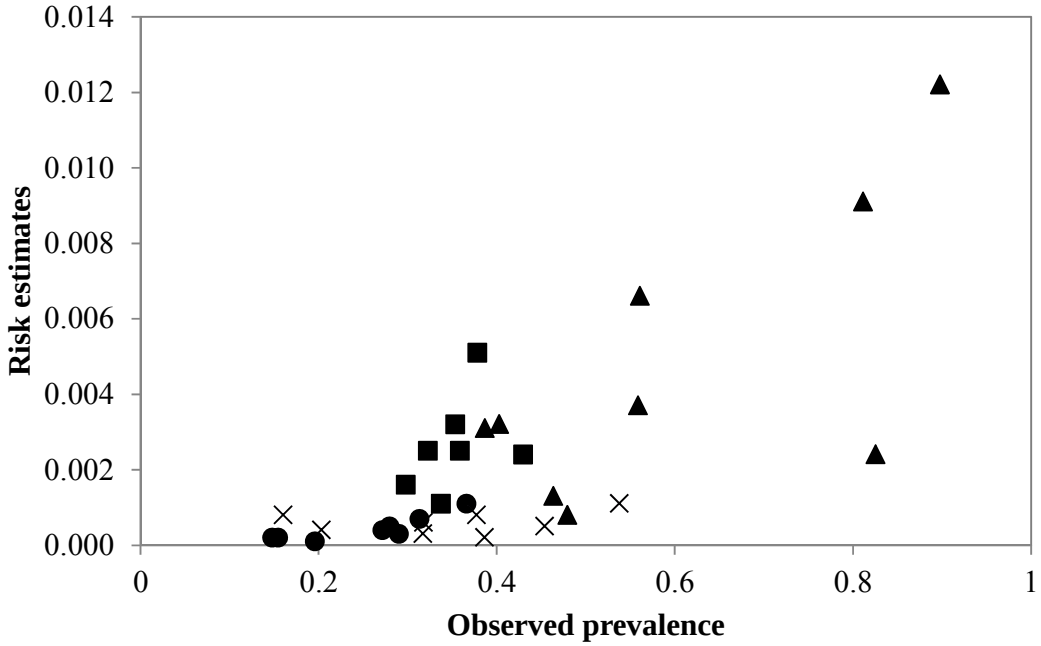


Figure 6


0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
Risk estimates
0
0.2
0.4
0.6
0.8
1
Observed prevalence

Manuscript VI

Source Attribution of Human Campylobacteriosis in Denmark

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Key words: *Campylobacter*, source attribution, MLST, *flaA*

Abstract

The aim of the present study was to assess the relative contribution of different sources of human campylobacteriosis in Denmark using two different source attribution approaches able to identify the primary reservoirs of domestically acquired cases based on MLST sequence types and *flaA* types.

In total, 794 non-human isolates were collected in 2007 and 2008; comprising broilers, Danish and imported chicken meat, turkey meat, duck meat, cattle, and pigs. Within the same period, isolates from 406 human cases were collected (246 from domestic cases, 109 from travel related cases, and 51 from cases without travel history). All isolates were characterized by Multilocus sequence typing, *flaA* typing and susceptibility to antibiotics.

Danish broiler chicken was found as the primary reservoir for Danish campylobacteriosis cases. The Asymmetric Island model attributed the vast majority of cases to the broiler chicken reservoir; 52% (CI 37-67%) to Danish chicken and 17% (CI 3-33%) to imported chicken, while the second most important reservoir was cattle; 17% (CI 7-28%). Similarly, the CAMSA model apportioned 38% (CI 28-47%) to Danish chicken and 14% (CI 10-18%) to imported chicken, while sixteen percent of cases (CI 7-25%) were attributed to cattle. Approximately half of the cases related to the broiler chicken reservoir were considered to be associated with handling, preparation and consumption of broiler meat. The addition of the sequenced *flaA* gene, as an extra discriminating typing parameter, did not cause the attribution of cases markedly, but refined the apportioning of human cases further, especially with regard to discriminating between the chicken and cattle reservoirs.

Both models indicate that the major burden of human campylobacteriosis in Denmark originates from the domestic broiler chicken reservoir. The results of the attribution models provide risk managers with a new tool to support decision making in relation to intervention strategies.

Introduction

Campylobacter species continue to be a major problem in large parts of the world, including Denmark, being one of the most common causes of human bacterial gastroenteritis (1-3). The species most frequently associated with human disease are *Campylobacter jejuni* and *Campylobacter coli*. The proportion between the species varies between countries (2). In Denmark, the vast majority of human campylobacteriosis cases is caused by *C. jejuni* (approximately 96%) (4).

Campylobacter species have been detected in many sources and are considered to be widespread in production animals and in the environment (5). In many countries, travel is considered a major risk factor for acquiring campylobacteriosis (6). In Denmark, travel is estimated to account for approximately one third of the human campylobacteriosis cases (7). Broiler chicken meat is recognized as the largest single source of foodborne campylobacteriosis cases (6;8). Consequently, several countries have already established action plans against *Campylobacter* in the broiler production chain (9). Also in Denmark, several initiatives have been initiated to bring down the *Campylobacter* burden in the broiler production (10).

The Danish strategy to control *Campylobacter* has had a positive effect; reducing the prevalence in broiler flocks and broiler meat. A small decrease in the number of human cases has also been observed. The explanation for the effect on human cases not being more significant is probably due to other factors counterbalancing the effect of the implemented interventions (10). In particular, imported broiler meat and other sources of infection than broilers are assumed to have influenced the limited effect on humans. Consequently, it could be interesting to evaluate which other sources that potentially add to the total number of human campylobacteriosis cases. With regard to the Danish *Salmonella* situation, the development of a source attribution model proved to be an important tool for risk managers in order to implement targeted interventions, resulting in

significant reductions in human salmonellosis (11). It would be of great importance if a similar tool could be developed for *Campylobacter* for prioritization of public health resources and source specific implementation of control measures.

Several methods for source attribution are available (12), however, some are more evident to apply for *Campylobacter* than others. For example, analysis of outbreak data makes little sense, as outbreaks caused by *Campylobacter* are considered to be rare. Interesting results have been shown using the microbial subtyping approach; i.e. in New Zealand and England (13;14). The models used were modifications of the original Danish *Salmonella* model and the newly developed Asymmetric Island model (13;14). Both studies found poultry to be the main reservoir, and broilers to be the principal source of human campylobacteriosis caused by *C. jejuni*. Second to broilers came the cattle reservoir.

A challenge in source attribution for *Campylobacter* cases based on microbial subtyping is that the individual subtypes appear to be widespread between sources. The discriminatory ability of the commonly used typing schemes does at present not allow for a very distinct separation between sources, which complicates the attribution of human cases to possible sources. To be able to attribute human cases to different sources, some genetic diversity between source groups is essential. Future identification of more source-specific attributes would be of great importance for optimizing the apportioning of cases.

With the perspective of the potential benefit from adaption of a *Campylobacter* source attribution tool with regard to risk management, the aim of the present study was to assess the Danish *Campylobacter* situation using two different source attribution approaches based on MLST sequence types of *C. jejuni* isolates; each approach identifying the primary reservoirs of domestically acquired cases and cases with unknown travel history. Furthermore, we explore the effect of adding an extra typing parameter (the *flaA* gene) for additional discriminatory power.

Materials and Methods

Isolates

C. jejuni isolates included in the study were collected in the years 2007 and 2008 and originated from various projects including the EU baseline study of broiler carcasses (15), the national *Campylobacter* surveillance for broiler meat (at slaughter plants and at retail), and the national surveillance for antimicrobial resistance (including various animal species) (16). Some isolates were collected for this study only.

In total, 406 human isolates were collected from three regions of Denmark: Northern Jutland, Funen and Zealand. Of the human isolates, 246 were reported as domestic (i.e. acquired in Denmark), 109 were reported as travel related, and 51 were with unknown travel history. Cases were reported as related to travel, if a person in the seven days period prior to disease onset had stayed a minimum of one night in any other country than Denmark (7). All human isolates were from cases characterized as sporadic i.e. not associated with any known outbreaks. Isolates from six putative sources were included: Danish broiler meat (185 isolates collected at retail), imported broiler meat (137 isolates collected at retail), turkey meat (96 isolates collected at retail), duck meat (70 isolates collected at retail), cattle (171 isolates from faeces), pig/pork (3 isolates from caecum and 1 isolate collected at retail).

Multilocus Sequence Typing (MLST). flaA typing and antimicrobial susceptibility testing

All isolates were characterized by MLST sequence type, *flaA* type and antimicrobial susceptibility.

MLST typing were carried out according to the scheme described by Dingle *et al*. (17); sequencing seven housekeeping genes. For each house-keeping gene, the different sequences were assigned as distinct alleles (by assignment a single number) and, for each isolate, the alleles at each of the seven loci defined the allelic profile, which determined the sequence type (ST).

The *flaA* type was determined by PCR amplification, DNA sequencing and alignment as previously described by Meinersmann *et al*. (18). The PubMLST database was used for identification of profiles (http://pubmlst.org/campylobacter/).

Antimicrobial susceptibility was determined as described in DANMAP 2008 (16). In brief, the antimicrobial susceptibility testing was performed by microbroth dilution MIC with the Sensititre system (Trek Diagnostic Systems Ltd., UK). Inoculation and incubation procedures were in accordance with CLSI guidelines. MIC values were interpreted using EUCAST epidemiological cut-off values. The susceptibility was determined for the following antimicrobial agents: chloramphenicol, ciprofloxacin, erythromycin, gentamicin, nalidixic acid, streptomycin, and tetracycline. The result of the testing was coded as resistant (R) or sensitive (S). An isolate was categorized as resistant if resistant to at least one of the specified agents.

Analysis of Molecular Variance (AMOVA)

The genetic structure differentiation between groups was tested by AMOVA (19). AMOVA is a method of testing population differentiation directly from molecular data, based on Euclidean distance metrics. Variance components are used to calculate statistics called Φ-statistics, summarizing the degree of differentiation between population groups. The genetic distance between a pair of isolates was defined as the number of loci, out of seven, at which they differed. Analysis of molecular variance was carried out in the Arlequin software version 3.11. The number of

permutations for significance was 999 and the level of significance was set to 0.05. The genetic diversity between groups (Φ_{GT}) was tested by estimation of the pairwise difference. The lower the value of Φ_{GT}, the lesser the variation between groups. A significant *p*-value indicates statistical difference between groups.

Source attribution modeling

Two models were used to attribute human cases: The Asymmetric Island model (AI model) developed by Wilson et al. (13) and a model modified after the Danish *Salmonella* attribution model (20). The second model will be denoted the CAMSA (CAMpylobacter Source Attribution) model in the following.

The AI model apportions domestic cases to different putative sources defined by relatedness to groups comprising isolates collected from the respective source. This model is an evolutionary model taking into account mutation, recombination and migration rates. The model was used as it is without modifications. Convergence of the model was monitored by multiple runs ensuring equal results; 100,000 iterations were run without thinning. The model was run through the freely available software iSource, which was downloaded from the website: http://www.danielwilson.me.uk/software.html. The input for the model was the allelic profile of each isolate in the source groups. For attribution, domestic human cases and human cases with unknown travel history were used as input. In addition to running the model with the animal-food sources, it was further explored to run the model with the inclusion of a "source" called travel. Based on the 109 human cases categorized as travel related, an additional source was created. This was to investigate the possibility of allowing cases without travel history to be attributed to travel

and not making the assumption that these cases are all domestic or choosing not to attribute the cases at all.

The CAMSA model was adapted after the Danish *Salmonella* attribution model (20). This model apportions human cases using a Bayesian framework. The modeling was based on the occurrence of types in included sources, combined with the amount of the food source available for consumption and two factors regarding 1) the type specific ability to cause infection, and 2) the source specific ability to serve as a vehicle for the types. The equation used to estimate the expected number of human cases was:

$$\lambda_{ij} = M_j p_{ij} q_i a_j,$$

where λ_{ij} is the expected number of cases of type i from the source j, M_j is the amount of source j available for consumption, p_{ij} is the number of isolates of type i in source j (modification from the original model), q_i the sequence type dependent factor, and a_j the source dependent factor. The equation represents a multi parameter prior, where q_i and a_j are parameters of unknown value. These parameters were included as distributions; a hierarchical prior (modification from the original model) and a uniform prior, respectively. The use of a hierarchical prior was adapted after Mullner *et al*. (21), using a lognormal distribution $N(0,\tau)$. The prior distribution for τ was *gamma* (0.01, 0.01). The source dependent factor, a_j, was assumed equal for Danish produced and imported chicken meat. A Markov Chain Monte Carlo simulation, specifically the Gibbs sampler, was applied to compute the posterior distributions for a_j and q_i. Five independent Markov chains of 40,000 iterations were run. Convergence was monitored using the methods described by Gelman and Rubin (22). The model was run in WinBUGS version 1.4. The input for the model was the sequence types (STs). The ten STs most frequently found in humans were further differentiated by supplementing the STs with information of antimicrobial susceptibility. Domestic human cases and

human cases with unknown travel history were inputted for attribution. Cases categorized as related to travel were directly assigned as this.

The main difference between the two models is the principle behind attribution of cases and the factors which the models account for. The AI model is estimating the probability of each source for each human isolate, accounting for evolutionary relationship, meaning that all human cases will be apportioned on sources. The CAMSA model estimates the expected number of cases per source based on comparison of the observed number of cases caused by a specific type with the occurrence of types in specified sources, weighted by amount of food source available for consumption and accounting for type and source specific ability to cause disease. The CAMSA model requires an exact type match between isolates from humans and sources. Therefore, human types that are not found in any source are referred to the group "unknown".

Both models were run at two different discriminatory levels: level 1) differentiation based on the seven housekeeping genes (considered as the basic models), and level 2) differentiation based on the seven housekeeping genes + the *flaA* type.

Results

The results of the AMOVA analysis (Table 1) showed that the genetic differentiation between source categories was statistically significant for almost all groups. The genetic difference was not significant between the group comprising the domestic human cases and the group of human cases with unknown travel history. This indicates that human cases with unknown travel history were more similar to the group of domestic cases compared to the group of travel related cases. The pig-group was not statistically different from three of the groups, which was probably due to the very small number of isolates (N=4).

The output from the basic AI model is presented in Table 2. The model attributed the vast majority of cases to the broiler chicken reservoir; 52% (CI 37-67%) to Danish chicken and 17% (CI 3-33%) to imported chicken. The second most important reservoir in relation to human illness was cattle; 17% (CI 7-28%). The uncertainty around the attribution estimates ranged widely. This is influenced by the fact that most cases could not stringently be assigned to one particular source. In particular, the distinction between broiler chicken and cattle was vague (FIG 1A). The inclusion of travel as a "source" for cases with unknown travel history in the AI model only changed the attribution of cases very little. The vast majority of cases were still attributed to broiler chicken and cattle (Table 2). Approximately 11% (CI 1-29%) of cases with unknown travel history was attributed to travel; 2% of all apportioned cases.

For the CAMSA model, the sequence type dependent factor (q_i) was fairly equal between STs. Only the estimate for one ST (ST 4811) tended to be higher than the rest indicating this type to result in relatively more human cases as compared to the other STs. The food related factor (a_j) for cattle tended to be higher compared to the food related factor for other sources. The output of the CAMSA model was very similar to the results of the AI model (Table 3). The primary reservoir

being broiler chicken, comprising Danish chicken 38% (CI 28-47%) and imported chicken 14% (CI 10-18%). Sixteen percent of cases were attributed to cattle. Approximately one fifth of cases could not be assigned to any of the sources.

Both models attributed the majority of cases to the broiler chicken reservoir. Hereof, the Danish broiler chicken was found to be the largest contributor compared to imported chicken. The cattle reservoir was found to be the second most important in relation to human campylobacteriosis (Table 3).

Inclusion of the sequenced *flaA* gene caused different impacts on the attribution outputs from the two models (Table 3). For the AI model, the inclusion of the *flaA* gene boosted the proportion of cases attributed to chicken at the expense of the proportion attributable to cattle. The uncertainty around the attribution estimates still ranged wide, but diminished slightly following the inclusion of *flaA*. The inclusion of *flaA*, resulted in a larger proportion of cases more strictly associated with chicken (FIG 1B) compared to the basic model (FIG 1A). For the CAMSA model a larger proportion of cases fell in the group “Unknown” including a proportion from each group except travel. The proportion of cases attributed to cattle decreased slightly more compared to the other groups.

Discussion

Both models agreed in recognizing broiler chicken as the primary source of human campylobacteriosis. This supports the original hypothesis of chicken being the most important single source of human campylobacteriosis. A higher proportion of cases were apportioned to Danish chicken compared to imported chicken. Several factors can explain this. Firstly, the risk from Danish chicken might actually be higher compared to imported chicken. Secondly, the Danish broiler chicken reservoir comprise more transmission routes compared to imported chicken, as the production is taking place in the country of concern. Besides meat, also animal contact through for instance occupation may be a risk factor. Thirdly, some of the cases assigned to Danish chicken might actually belong to another reservoir, as the STs comprising the Danish chicken reservoir are more closely related to the other sources than the STs comprising the source of imported chicken. A Danish case control study found a population attributable risk from fresh chilled chicken of 24% of domestically acquired cases (8). A Scientific Opinion from EFSA suggests that handling, preparation and consumption account for only 20-30% of human cases while 50-80% may be attributed to the broiler chicken reservoir as a whole (6). This agrees very well with the Danish studies; attributing about 50-60% of cases to the broiler chicken reservoir as a whole with approximately half of these acquired from chilled chicken meat (8). It is not possible from the source attribution to estimate the proportion of cases caused by the handling, preparation and consumption of Danish chicken meat because the isolates collected represent all transmission routes from the chicken reservoir to the consumer. This is in contrast to the imported chicken meat, where transmissions routes prior to packaging are of no risk for the Danes. However, we know that the proportion of Danish/imported meat available for sale is 60/40 (in 2008). Assuming no difference in

ability of infection between types and combining source estimates and consumption data, we could infer, that human cases caused by Danish chicken meat would be approximately 21% of all cases.

Cattle were found to be the second most important source. High *C. jejuni* prevalence has been reported in cattle (23;24), however very low occurrence has been found in beef (23;24). If cattle should bear the second highest responsibility in relation to human campylobacteriosis, routes other than meat should probably be regarded. This would agree with the results from a Dutch comparative exposure assessment ranking farm animal contact higher compared to beef with regard to importance of transmission (25). The *Campylobacter* occurrence in meat from ducks is high in Denmark (1;26). However, only few cases were attributed to this reservoir by the models. The reasons for this observation is probably the way of handling and preparing this product and that the consumption of this type of meat are smaller compared to chicken meat. Traditionally, ducks are prepared whole and hours before the garnish reducing the risk of cross-contamination considerably. The proportion of cases attributed to turkey, being smaller than broiler chicken and higher than duck, fits with the proportionate consumption of turkey meat being lesser than chicken and higher than duck.

The addition of travel-related cases as a "source" in the AI model estimated approximately 11% of cases with an unknown travel history to this group. This estimate corresponds to what was found with the CAMSA model and also the result of the AMOVA analysis; finding a greater similarity between cases without travel history and domestic cases as oppose to travel-related cases. The introduction of travel in the AI model is a way to cope with the fact that information about travel history might not always be available for all human cases. This scenario is probably the situation in many countries as it is not always feasible to obtain this information. As travel is considered an important risk factor for acquiring campylobacteriosis, the modeling could benefit from being able to handle this in case of missing travel information from some human cases.

The assumption that human cases that have been travelling one week prior to onset of illness have been infected abroad might not be true for every case. In Denmark, cases are reported as related to travel, if a person on the day of or seven days prior to illness onset has stayed for a minimum of one night in any other country than Denmark (7). However, there is still a possibility that the infection has been acquired in Denmark. Consequently, the estimate might be too high. In addition, the risk of acquiring campylobacteriosis varies between countries (7;27), which is not considered in the model at present.

The isolates representing each source were collected through different monitoring programs and surveys, which varied in sampling size, sampling period, etc. To achieve the best possible attribution, the variation in STs within sources should be covered. According to rarefaction analysis, this was not the case (Jonas Larsson, SSI, personal communication). With the exception of cattle, only a minor proportion of the full genetic diversity was covered by the isolates included in the project. An additional large number of samples would be needed to cover the huge variety in STs because of the great heterogeneity in *Campylobacter,* which would require an even larger number of samples to be collected. This work would be extremely costly.

Genetic diversity was found between isolates collected from chicken meat at retail and live broilers (Table 1). This might reflect that we have not covered the whole genetic diversity or that a potential natural selection of types through the processing chain might take place. We chose to use isolates from retail chicken to represent the Danish broiler chicken reservoir. The use of retail isolates as oppose to isolates from live broilers might to some degree reduce the bias of environmental types in the Danish broiler chicken reservoir.

Data from other sources were sampled but not included in the study. Samples were collected from 125 pet dogs and cats, however, only one *C. jejuni* isolate was found. It was decided not to include this isolate. Furthermore, samples from fresh water streams, petting zoo goats, milk cattle,

and meat from lamb were collected but unfortunately lost. We did also not collect samples from tap water, as this is not considered a source of sporadic cases in Denmark. The influence of these decisions and events may have resulted in less accuracy in assigning cases, as some cases might rightfully belong to the sources not included in the model. In theory, a larger part of the proportion of the group "unknown" from the CAMSA model may have been explained by the addition of more sources and the AI model might have attributed cases differently. Almost all tap water in Denmark is attained from the groundwater reserve and is filtered during diffusing from the surface through the soil layers. This is considered to be a sufficient hygienic barrier and no further treatment is done. Water quality is monitored by an extensive testing for indicator bacteria, but only sparse data exists for the actual occurrence of *Campylobacter* (28) and furthermore, the relation between *Campylobacter* and the indicator bacteria have not been confirmed. Waterborne outbreaks caused by *Campylobacter* have been observed in Denmark as result of contamination of the tap water supply and contamination of costal water after heavy rainfall (1), however, as a source of sporadic cases, drinking water is disregarded. Puppies, as opposed to older dogs have been found to excrete *C. jejuni* in Denmark as well as in other countries (29-31). Therefore, the young pets might pose a risk, which was not reflected by our sampling, as our sampling was not targeted towards puppies and kittens, but dogs and cats in general. Finally, a study by Evers *et al*. (25) demonstrated the potential importance of petting zoo animals. Further, there might remain other, not recognized sources.

Hence, the inclusion of all relevant reservoirs in modeling is important for the "correct" attribution of cases. Inclusion of more sources would probably not reduce the uncertainty in the attribution estimates, because of the great variation in STs within sources and the overlap in genotypes between sources would still exist and result in lack of clear separation. Even though MLST is highly discriminatory in typing *C. jejuni*, MLST cannot distinguish very clearly between

animal reservoirs. Numerous types are detected in several reservoirs; i.e. ST21 and ST45. A more nuanced attribution of cases could be obtained in case of identification of more source specific attributes. In addition to performing source attribution based on only the MLST data, and resistance profiles for the CAMSA model, the potential in adding another attribute, in this case the sequenced *flaA* gene, was explored. For the CAMSA model, this addition decreased the number of cases that the model was able to assign. This model is seeking exact matches between human and source types, why the added discriminatory power results in fewer matches between cases and sources. The proportion of cases attributed to the different sources decreased in approximately equal magnitude, suggesting that additional sampling of sources are needed to cover the large variation in STs. Only the proportion assigned to cattle decreased slightly more. For the AI model and as illustrated in Figure 1 A and B, the discriminatory power was increased by the addition of the *flaA* gene. Especially with regard to differentiation between Danish broiler chicken and cattle the addition appeared valuable.

There are pros and cons in using either of the attribution models. Both models need a considerable amount of data. The AI model infers the apportioning of cases based on the available sources, accordingly producing estimates for only the implicated sources. This might skew the results if not all putative sources are represented in the data. The CAMSA model, on the other hand, has a category (unknown) for the cases that cannot be attributed to one of the sources in the model, however, requires an exact match of ST to assign cases to a source. Both ways to handle the data can be positive as well as negative. To attribute every case to a source might wrongly boost some categories if not all putative sources are included in the model, however, only to attribute cases with exact ST match might be too stringent. Especially for *Campylobacter*, a close relatedness can be sufficient and reduce the huge number of samples per source that should be collected to cover the

great variation of types encompassed within the sources. Overall, the two models were considered to complement each other.

The use of a reservoir model might produce different results than for example a model attributing cases at the point of consumption (i.e. comparative exposure modeling). For example, attribution of *Campylobacter* infections may assign a larger proportion of cases to chicken at reservoir level than to chicken meat at the point of consumption. For example, if raw vegetables were cross-contaminated during processing or preparation in the kitchen, the source at the point of consumption would be the vegetables while the reservoir would be broiler chicken. The application of a reservoir model could be beneficial with regard to interventions within the domestic productions systems. This enables preventing the problem at its root, as it is difficult to influence consumer behavior, such as cross-contamination in the kitchen (32). Furthermore, attribution at the reservoir level disregards the comprehensive network of transmission routes. On the other hand, modeling source attribution, for example, at the point of consumer purchase, might reveal problems within production chains; i.e. contamination of ready-to-eat vegetables, which was potentially not contaminated in the primary production. The latter approach requires inclusion of yet further sources, meaning large amount of data and thereby considerably resources. The availability of data to fulfill the requirement to do a reasonable exposure assessment for source attribution purposes is questionable. In the essences, it would be optimal to control the organism at the starting point if possible.

Substantial work has been carried out in New Zealand in the fight against *Campylobacter*. Included in this work was the use of source attribution for identification of specific sources for human campylobacteriosis (caused by *C. jejuni*). The initial source attribution work found that 58-76% of human cases was caused by poultry sources (14). Similar results was found in England, estimating chicken as a source of human illness in 51-62% of cases (13). These findings are similar

to the results from the present work on *Campylobacter* source attribution in Denmark. After implementation of a comprehensive intervention strategy, an evaluating source attribution work revealed a decline in the number of cases attributed to poultry in New Zealand by 74% (33). No dramatic decrease has been observed in human cases in Denmark after the implementation of *Campylobacter* intervention strategies. The adaption of source attribution could be a valuable tool to assess present and future intervention strategies. As the Danish source attribution points at the broiler chicken reservoir, in particular the Danish broiler chicken, as being the most important for human campylobacteriosis cases, it must be recommended to evaluate the implemented interventions as a logical next step, to evaluate their efficacy. However, when comparing the results obtained in New Zealand, the difference in food trade patterns should be considered; New Zealand having no import of chicken meat as oppose to Denmark where about 40% of the meat available for consumption is imported (in 2008). This of course affects the potential of national action plans to be effective.

Other studies have found that children below the age of five are becoming ill from different sources compared to older population groups and also living in rural as oppose urban areas influence the epidemiology (34;35). In future studies, we will look at the distribution among age groups and rural versus urban areas. Furthermore, it could be of particular interest to try to combine the source attribution using microbial subtyping with comparative exposure assessment, and hereby adding information of the plausibility of *Campylobacter* reaching the consumer via the various transmission routes.

Conclusions

Both models applied MLST typing data for source attribution and indicated that the major burden of human campylobacteriosis in Denmark originates from the broiler chicken reservoir. This was further emphasized by applying additional discriminatory power to the models by including *flaA* subtypes. Using source attribution as basis for national interventions for *Campylobacter* has proven effective in New Zealand. The results of the present study can be useful for future risk management decisions related to the control of *Campylobacter* in Denmark.

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Disclosure Statement

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Figure legends

Figure 1. Probability of each human case belonging to each of the included sources (results from the basic AI model). A) Modeling based on MLST types, B) modeling based on MLST types + *flaA*. The probability is depicted by color coding ; cattle (dark blue), Danish chicken (red), imported chicken (yellow), turkey (green), duck (cyan), pork (pink).

Tables

Table 1. Genetic differentiation between groups. Below the diagonal is the estimated pairwise difference between groups (Φ_{GT}), above the diagonal is the associated *p*-value.

Pairwise difference (Φ_{GT}) /p-value	Human (travel related)	Human (domestic)	Human (unknown)	Broilers	Chicken DK	Chicken IMP	Turkey	Duck	Cattle	Pig
Human (travel related)	*	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	**0.164**
Human (domestic)	4.6%	*	**0.106**	0.000	0.000	0.000	0.000	0.000	0.000	0.001
Human (unknown)	3.2%	**0.1%**	*	0.003	0.005	0.003	0.001	0.000	0.000	0.044
Broilers	6.2%	4.5%	2.7%	*	0.007	0.000	0.000	0.000	0.000	0.000
Chicken DK	3.1%	1.9%	0.6%	0.9%	*	0.000	0.000	0.000	0.000	0.000
Chicken IMP	1.3%	2.9%	2.0%	4.5%	2.4%	*	0.000	0.000	0.000	0.050
Turkey	3.4%	5.8%	3.5%	2.5%	2.0%	2.5%	*	0.000	0.000	**0.517**
Duck	15.0%	15.3%	13.1%	10.8%	11.8%	10.8%	6.5%	*	0.000	**0.116**

Cattle	5.5%	3.9%	1.9%	7.0%	5.0%	4.3%	7.0%	16.1%	*	0.001
Pig	**21.6%**	25.3%	20.9%	20.9%	21.7%	20.2%	**18.3%**	**24.5%**	19.9%	*

Non-significant Φ-statistics printed in bold

Table 2. Attribution of human cases with the AI model, excluding and including travel as a "source". Proportion of cases attributable to the specific source and corresponding uncertainty (CI 95%[a])

	AI						
	All cases			Domestic cases		Unknown travel history	
	Excl. travel	CI 95%	Incl. Travel[b]	Excl. travel	CI 95%	Incl. travel	CI 95%
	N=297		N=297	N=246		N=51	
Chicken DK	0.52	0.37-0.67	0.49	0.52	0.36-0.67	0.33	0.12-0.56
Chicken IMP	0.17	0.03-0.33	0.16	0.17	0.03-0.32	0.15	0.01-0.36
Turkey	0.05	0.00-0.24	0.05	0.05	0.00-0.23	0.07	0.00-0.23
Duck	0.02	0.00-0.10	0.03	0.02	0.00-0.11	0.05	0.00-0.15
Cattle	0.17	0.07-0.28	0.18	0.17	0.07-0.28	0.23	0.07-0.42
Pig	0.07	0.00-0.18	0.07	0.07	0.00-0.18	0.06	0.00-0.20
Travel	-	-	0.02	-	-	0.11	0.01-0.29

[a] Credibility interval

[b] Based on the separate models for domestic cases and cases without travel history

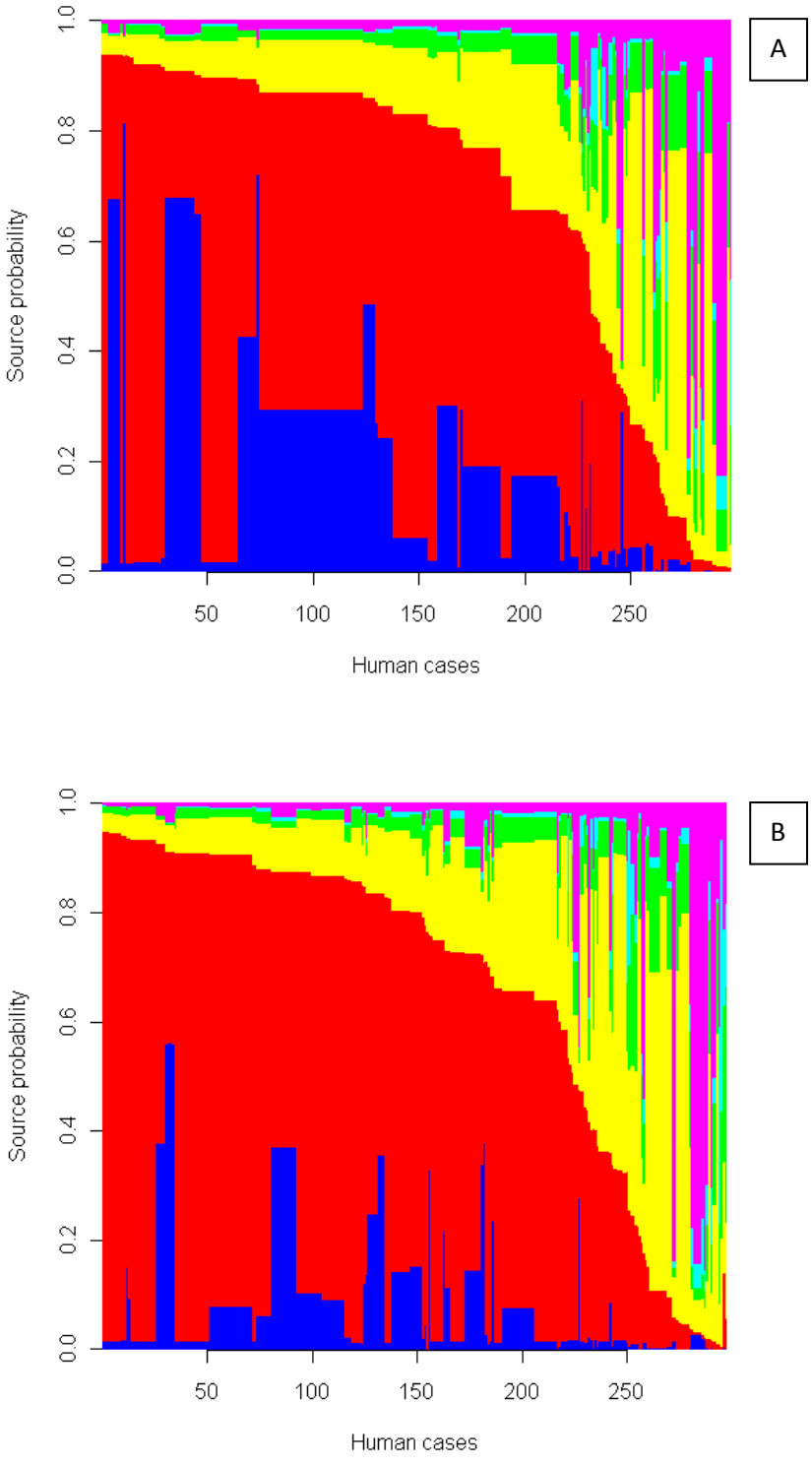
Table 3. Proportion of cases attributable to the specific source and corresponding uncertainty (CI 95%[a]). Attribution of human cases (domestic and without travel history) with basic models; differentiation based on MLST sequence types (Input 1) and differentiation based on MLST sequence types + the typed *flaA* gene (Input 2)

	CAMSA				AI			
	Input 1	CI 95%	Input 2	CI 95%	Input 1	CI 95%	Input 2	CI 95%
Chicken DK	0.38	0.28-0.47	0.35	0.27-0.43	0.52	0.37-0.67	0.57	0.41-0.72
Chicken IMP	0.14	0.10-0.18	0.12	0.09-0.15	0.17	0.03-0.33	0.19	0.03-0.37
Turkey	0.06	0.01-0.13	0.06	0.02-0.12	0.05	0.00-0.24	0.06	0.00-0.22
Duck	0.03	0.01-0.06	0.04	0.01-0.08	0.02	0.00-0.10	0.02	0.00-0.09
Cattle	0.16	0.07-0.25	0.10	0.04-0.17	0.17	0.07-0.28	0.08	0.01-0.18
Pig	-	-	-	-	0.07	0.00-0.18	0.07	0.00-0.19
Unknown	0.21	0.10-0.31	0.32	0.22-0.41	-	-	-	-
Travel	0.03	0.02-0.04	0.03	0.02-0.04	-	-	-	-

[a] Credibility interval

Figures

Figure 1.



APPENDIX

Appendix Tables

Appendix Figures

Appendix table 1. Brief description of nationally implemented control measures described in published action plans to control *Campylobacter* in the domestic broiler production in Denmark, Iceland, Norway, New Zealand, and United Kingdom

	DK	IS	NO	NZ	UK
MANDATORY	No	Yes	Yes	No (Yes[a])	No
TARGETS	-	-	-	+	+
INTERVENTIONS					
Primary production	Biosecurity GMP[b] industry code of practice	Biosecurity Producer education	Biosecurity Producer education	Biosecurity Biosecurity	
Slaughterhouse	Freezing (to the extent possible)	Freezing (mandatory) Reduced slaughter age	Freezing/heat treatment (mandatory) Intensified GHP[c]	Intensified GHP[c]	
Consumer	Leak-proof packaging[d] Consumer campaigns	Leak-proof packaging Consumer campaigns	Leak-proof packaging	Leak-proof packaging Consumer campaigns	
STRATEGIES					
	First initiatives 1998 Strategy 2003-2007 Strategy 2008-2012	First initiatives 2000	First initiatives 2000 Strategy established in 2001, modified several times	First initiatives 2006 Strategy 2007-2010 Strategy 2008-2012	First initiatives 2001 Strategy 2010-2015
REFERENCE	*(manuscript I)*	*(117)*	*(66)*	*(110)*	*(9, 10)*

[a] Compliance with performance target

[b] Good Manufacturing Practice

[b] Highly focused on chemical decontamination

[c] Good Hygiene Practice

[d] not actively part of the action plan, but standard procedure for packaging

Appendix table 2. Surveillance of *Campylobacter* in production animals and food sources, 1995-2010

	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
ANIMALS																
Cattle	X	X	X	X	X	X			X	X	X	X	X			
Broilers	X	X	X	X	X			X	X	X	X	X	X			
Pigs	X	X	X	X	X	X			X	X	X	X	X			
Sheep					X											
Turkey					X	X	X	X	X	X						
FOODS																
Beef	X	X	X				X	X	X	X						
Broiler (retail)	X	X	X	X	X	X	X	X	X	X	X	X	X			
Broiler (abattoir)										X	X	X	X			
Duck														X	X	X
Game		X	X													
Pork	X	X	X				X	X	X							
Shellfish			X													
Turkey	X	X	X	X	X		X	X	X	X	X	X	X			
Fruits			X													
Vegetables			X				X	X	X							X

Appendix table 3. Describing the registered number of human cases including 95% confidence intervals, *Campylobacter* prevalence in broiler flocks, *Campylobacter* prevalence in broiler meat sampled at retail including 95% confidence intervals, and estimates of relative human risk of campylobacteriosis from Danish and imported meat as a sum of the risk from chilled and frozen meat (taking into account proportion of sale)

				Prevalence in retail meat										
Year	Human	95%-CI	Prevalence broiler[1]	DK chill	95%-CI	DK frozen	95%-CI	IMP chill	95%-CI	IMP frozen	95%-CI	RR DK meat	RR IMP meat	RR total
2001	4,620	4,487-4,753[a]	41.8	33.7	29.5-38.0	13.9	9.7-18.1	38.7	31.8-45.5	38.6	30.2-47.1	0.26	0.26	0.52
2002	4,379	4,249-4,509[a]	42.6	43.0	35.9-50.0	15.2	8.8-21.6	80.2	72.8-87.5	35.0	16.0-54.1	0.55	0.36	0.91
2003	3,536	3,419-3,536[b]	34.2	32.1	37.2-60.8	19.6	10.1-24.5	81.2	49.4-72.3	16.0	9.3-22.8	0.66	0.54	1.19
2004	3,724	3,604-3,844[bd]	27.0	38.5	26.9-50.1	14.8	10.6-19.0	82.6	71.3-90.9	31.7	22.2-41.2	0.35	0.29	0.64
2005	3,677	3,558-3,796[bd]	30.4	37.8	32.3-43.4	27.2	23.3-32.5	89.8	85.7-93.9	53.8	47.5-62.7	1.06	1.31	2.37
2006	3,242	3,130-3,354[c]	30.8	26.9	17.2-36.7	15.4	12.3-18.6	56.1	51.9-60.3	45.4	40.5-50.3	0.26	0.92	1.18
2007	3,861	3,739-3,983[d]	26.8	32.3	26.6-37.9	31.3	25.2-37.4	55.9	50.0-61.8	37.7	31.9-43.5	0.66	0.34	1.00
2008	3,455	3,340-3,570[bc]	26.3	29.8	27.3-32.3	28.0	17.2-24.8	40.3	37.2-43.4	31.8	26.8-36.8	0.53	0.50	1.03
2009	3,352	3,239-3,465[bc]	29.4	35.9	32.6-39.2	29.0	24.9-33.1	46.4	42.8-50.0	20.3	16.9-23.8	0.60	0.29	0.89
2010	3,949	3,826-4,072[d]	NA[2]	35.4	32.0-38.7	36.6	32.5-40.8	48.0	43.9-52.0	32.0	25.5-38.6	0.83	0.31	1.14

[1] Calculation of prevalence is based on the whole broiler population

[2] Changed sampling

Appendix table 4. Descriptive characterization of isolates[a]

Human isolates	Number of isolates	Identified STs	Found in sources: STs	Found in sources: Number of isolates	Not found in sources: STs	Not found in sources: Number of isolates
Domestic aquired	246	70	35	199	35	47
Travel related[b]	109	59	14 41 45 68			
Travel history unknown	51	32 18 36 14 15				

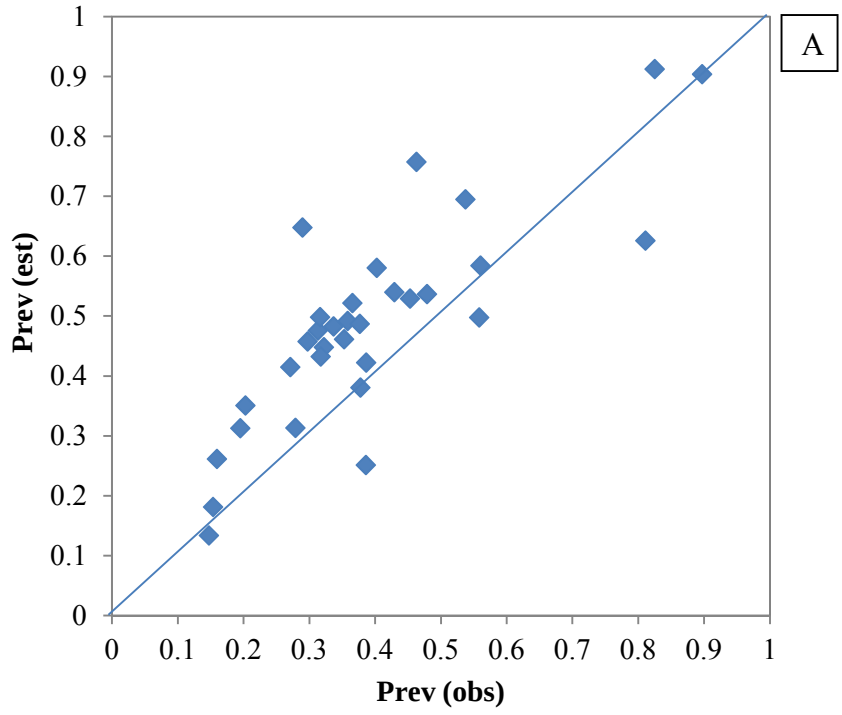
Source isolates	Number of isolates	Identified STs	Found in humans: STs	Found in humans: Number of isolates	Not found in humans: STs	Not found in humans: Number of isolates
Broilers[c]	189	68	28	135	40	54
Danish chicken	185	54	32	146	22	39
Imported chicken	137	64	26 87 38 50			
Turkey	96	60 19 41 41 55				
Duck	70	51 13 18 38 52				
Cattle	171	30	15	139	15	32
Pig	4	4 0 0 4 4				

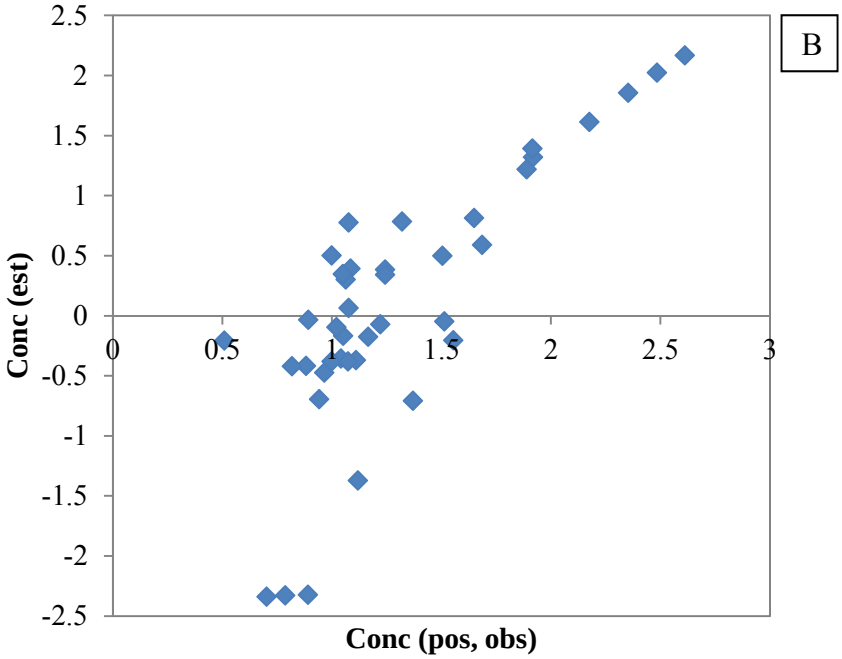
[a] Differentiation based on Multilocus sequence typing

[b] Human cases that have been travelling one week prior to onset of illness were characterized as related to travel

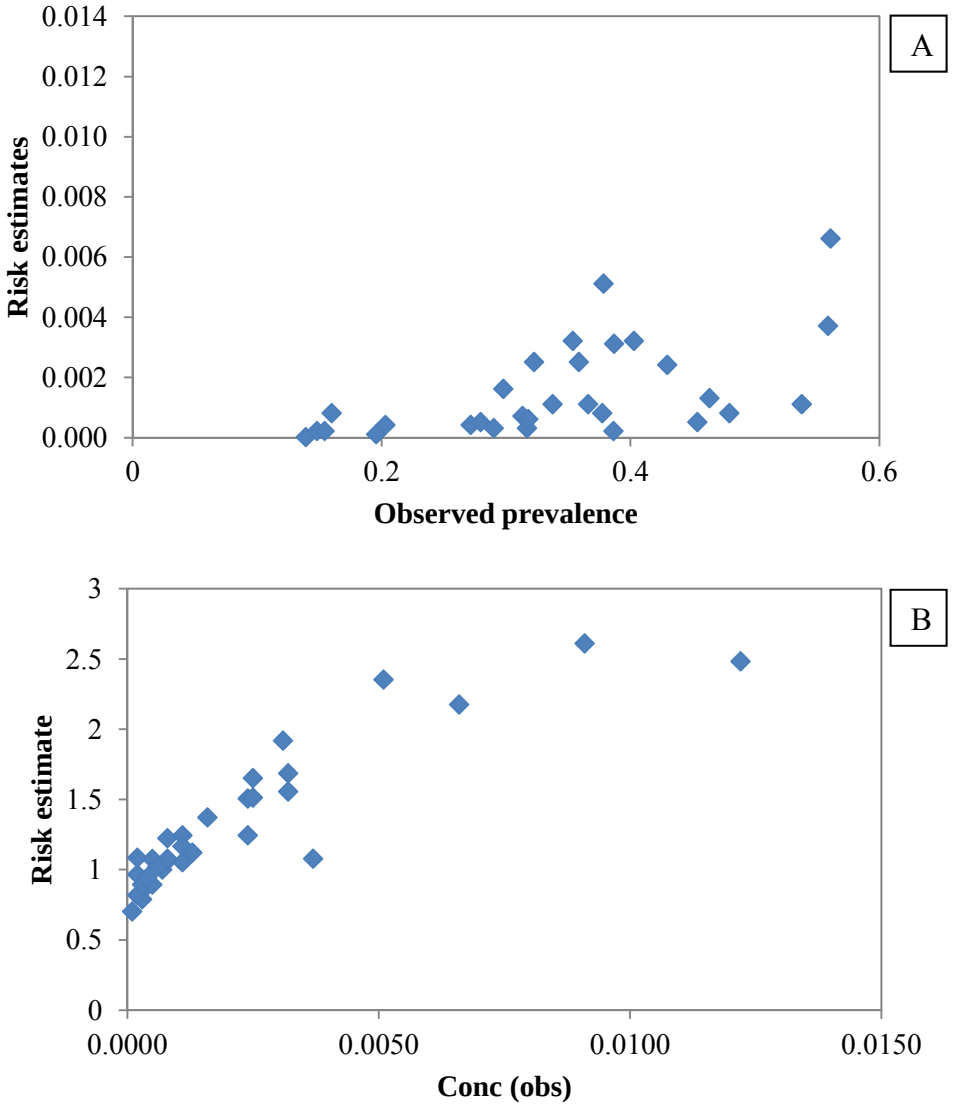
[c] Broiler isolates not included in the source attribution modelling

Appendix figure 1. Correlation between observed data versus maximum likelihood estimates. Data points representing estimates for all meat categories (Danish and import, chilled and frozen) for all years, 2001-2010. A) MLE fitted prevalence estimates (P_{est}) versus the observed prevalence (P_{obs}) (quarterly means), B) MLE fitted estimates of mean concentration versus observed mean concentration (calculation based on the highest number within the semi-quantitative intervals for each positive sample)

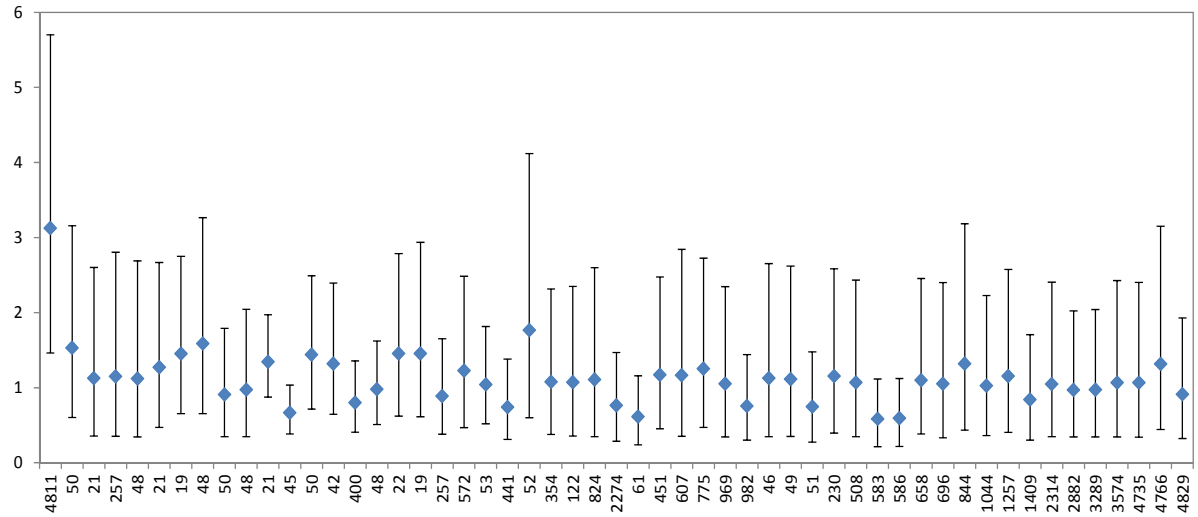




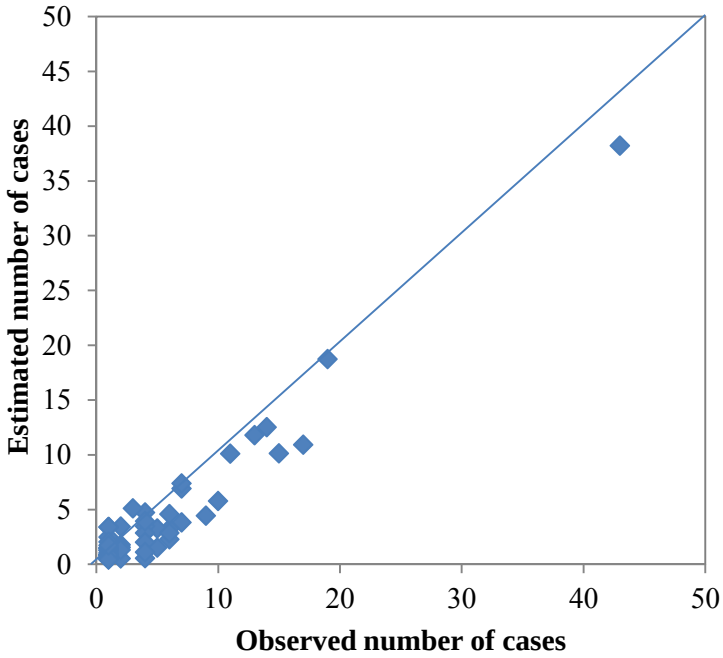
Appendix figure 2. Correlation between observed data versus risk estimates. Data points representing estimates for all meat categories (Danish and import, chilled and frozen) for all years, 2001-2010. A) Risk estimates versus the observed prevalence (quarterly mean), B) risk estimates versus the observed mean concentration (calculation based on the highest number within the semi-quantitative intervals for each positive sample)



Appendix figure 3. Estimated subtype related factors (q_i) for the standard model, based on MLST types (mean and 95% credibility interval)



Appendix figure 4. Plot of the observed number of cases (o_i) against the expected number of cases (λ_i) for individual sequence types obtained with the CAMSA model



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