The interaction between human antimicrobial use and the risk of foodborne zoonotic bacteria

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Publication date:
2014

Document Version
Peer reviewed version

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PhD Thesis

2013

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The PhD scholarship was supported partly by the EU Marie Curie Programme (MEST-CT-2004-007819) and the Danish Food Industry Agency (grant number 3304-FVFP-07-721-01).

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Summary

Salmonella enterica, *Campylobacter jejuni* and *Campylobacter coli* are the most common causes of foodborne bacterial infections worldwide. Both bacterial species have many modes for transmission in the food chain through which humans can be infected. The widespread use of antimicrobial drugs for food animals and the consequent dissemination of antimicrobial drug resistance have been well described in literature. Much less investigated is the association between human antimicrobial drug use and the adverse consequences it may have on human infections.

This thesis addresses the relation between antimicrobial drug use in humans, and the acquisition of infection with antimicrobial resistant non-typhoidal *Salmonella, Campylobacter coli* (*C. coli*), and *Campylobacter jejuni* (*C. jejuni*). The main objectives were:

1) To assess if the history of human use of antimicrobial drugs is a risk factor for acquiring infection with an antimicrobial *Salmonella* or *Campylobacter* strain.

2) To compare clinical outcome of disease for patients infected with *Salmonella* Typhimurium having different antimicrobial susceptibility profiles (i.e. pansusceptible, resistant or multidrug-resistant).

3) To examine how clinical outcome of an infection is affected by previous antimicrobial exposure.

A general overview of the discovery of antimicrobials, and the development and mechanisms of antimicrobial drug resistance in *Salmonella*, *C. jejuni*¸ and *C. coli* are described in chapter 2. Several features of the epidemiology, sources of infection, antimicrobial resistance, and surveillance of *Salmonella* and *Campylobacter* are described in chapter 3 and 4, respectively.

The history of human use of antimicrobial drugs in relation to acquiring an infection with *Salmonella* or *Campylobacter,* and the subsequent risk of the causative pathogen being resistant to the drug taken previously and unrelated to the infection in question was assessed in **Manuscript I** and **Manuscript II**. Both studies had the same study design: registry based case-control study, for which several of the Danish registries were merged using the unique Civil Registration Number (CPR), and approximately ten controls were matched to each patient on sex, age, and county of residence. Data on history of antimicrobial use was derived from the National Prescription database; cases enrolled in the study were retrieved from the National Registry for Enteric Patients (NREP); the Integrated Database on Labour Market Research provided data on socio-demographics of cases and controls; and the Civil Registry System was used to derive the CPR numbers, date of birth, and residential area of cases and controls.

A total of 22,609 *Salmonella* cases that were laboratory confirmed between 1997 - 2005, were enrolled in the study. The analyses were performed separately for *Salmonella* Typhimurium (*S.* Typhimurium, 4,534 cases), *Salmonella* Enteritidis (*S.* Enteritidis, 4,195 cases), and all other *Salmonella* serotypes combined (5,776 cases). We found that treatment with trimethoprim, sulphonamides, broad-spectrum penicillins, tetracyclines and fluoroquinolones, during one year before diagnosis, was associated with an increased risk of non-typhoid *Salmonella* infection. Overall, the highest risk was associated with the prior use of fluoroquinolones. The risk increased as the time-window of exposure approached the infection date. The Odds Ratios (OR) for previous use of fluoroquinolones were OR 4.6 (95% confidence interval (CI): 3.8 – 5.5) for other *Salmonella* serotypes, an OR 2.2 (95%CI: 1.7 – 2.9) for *S.* Typhimurium, and an OR 2.1 (95%CI: 1.8 – 2.4) for *S*. Enteritidis. Additionally for fluoroquinolones, we found an interaction term for the pathogen being resistant to fluoroquinolones and a history of fluoroquinolone use; OR 3.6 (95%CI: 1.2

– 10.3) for *S.* Typhimurium and OR 2.7 (95%CI: 1.2 – 5.9). Meaning that the risk for being diagnosed with a fluoroquinolone resistant *S.* Typhimurium after treatment with this drug in up to a year before diagnosis was 7.2 (2.0*3.6) times higher for patients than for controls. For *S.* Enteritidis the corresponding risk was 4.5 (1.7*2.7) times higher for cases than for controls. These findings are ascribed to the competitive and the selective effect of acquiring antimicrobial resistance, respectively. The competitive effect occurs when a course of antimicrobials taken disrupts the natural barrier effect of the gut flora. The selective effect is an additional effect, occurring when a person is exposed to a pathogen resistant to the antimicrobial taken. This increases the risk of infection further due to the selective pressure put on other bacteria susceptible to the drug taken.

Between 1999 – 2005, a total of 31,699 cases of *Campylobacter* were laboratory confirmed in Denmark, and thus enrolled in the study. We found that being diagnosed with *Campylobacter* was associated with an increased odds of exposure to a course of fluoroquinolones, macrolides, broad spectrum penicillins, tetracyclines, and sulphonamides and trimethoprim, up to one year before onset of disease. The risk was highest for taking fluoroquinolones (OR 2.4, 95%CI: 2.0 – 3.0). Due to the low number of *Campylobacter* isolates being tested for other antimicrobial drugs than fluoroquinolones and macrolides, it was only possible to calculate the interaction term (or selective effect) for these two drugs. For fluoroquinolones, we found an effect modification of the strain additionally being resistant to the drug taken (OR 1.6, 95%CI: 1.1 – 2.3). The odds of being exposed to a course of fluoroquinolones was 2.4 times higher for cases diagnosed with a fluoroquinolone-sensitive *Campylobacter* than for controls whereas the odds of being exposed to a fluoroquinolone was 3.8 (2.4*1.6) times higher for cases with a fluoroquinolone-resistant *Campylobacter* than for controls. For macrolides, the interaction term was not significant (OR 1.0, 95%CI: 0.7 – 1.5). However, when we performed cubic spline plots of the OR of being exposed to a course of antimicrobials we found that being exposed to a course of macrolides provided a protective effect for being diagnosed with *Campylobacter*, up to one month before diagnosis. This effect is likely to be caused by the fact that the metabolites and active compound of macrolides are trapped into lysosomes of phagocytic cells, and get released at a very low rate and provide prolonged protection against invasive bacteria such as *Campylobacter*.

In **Manuscript III,** the relation between clinical outcomes of infection with *S.* Typhimurium and the antimicrobial resistance profile of the causative strain was assessed, together with the association between outcome of infection and previous antimicrobial use. A prospective case-case study was performed, using data obtained through telephone-conducted interviews, which were merged with data from the NREP and the Civil Registry System. Data were analysed using logistic regression. The interviews were conducted between January-June 2010, and a total of 150 *S.* Typhimurium cases were enrolled in the study. Cases were divided into three different groups according to the resistance pattern of the strain they were infected with: pansusceptible (S), resistant (R) to 1-3 antimicrobials, or multidrug-resistant (MR), i.e. resistant to 4 or more antimicrobials. We found that previous antimicrobial use, unrelated to the current *S.* Typhimurium infection, was associated with a higher odds of weight loss (OR 2.4, 95%CI: 1.1 – 5.5), hospital admission (OR 2.0, 95%CI: 1.0 – 4.1), and antimicrobial therapy for the current salmonellosis (OR 7.9, 95%CI: 2.8 – 16.8). The study focussed on short-term outcomes of disease (diarrhoea, nausea, etc.), and patients were interviewed relatively shortly after notification in the NREP. This may explain why this study, in contrast to other studies that focussed more on long-term outcome of disease (mortality, bacteraemia, etc.), did not find other more serious disease outcomes to be related to resistance profile. Also, it is possible that, due to our study design, we missed out on the most severely ill people, simply because they were too ill to participate in the interviews.

We also found that patients with a resistant (R) susceptibility profile had a higher odds of being hospitalised due to their salmonellosis (OR 2.5, 95%CI: 1.0 – 6.0), experience abdominal pain (OR 2.9,

95%CI:1.3 – 6.5), and feeling nauseated (OR 2.6, 95%CI: 1.1 – 6.2), than patients with a pansusceptible *Salmonella*. We found no increasing trend with increasing antimicrobial resistance (S versus MR). These findings may be an extension of the competitive and selective effect of antimicrobial treatment (**Manuscript I** and **Manuscript II**), where past antimicrobial treatment depletes or changes the composition of the gut flora in a way that increases severity of infection. Alternatively, a past history of treatment could be an indicator or proxy of a vulnerable patient.

The overall conclusion of this thesis is that human antimicrobial use interacts in many ways with the risk of being infected with antimicrobial-drug resistant strains of *Salmonella* and *Campylobacter, and that treatment with antimicrobials may be associated with severity of infection as well*. The protective role of macrolides as observed for *Campylobacter* infection adds another layer to the complexity of these interactions. Prudent use of antimicrobial drugs should always be advocated in human health practices. Future studies should point out whether the associations found in this thesis also applies to other pathogens.

Sammendrag

De fleste fødevarebårne bakterielle infektioner i Danmark og på verdensplan forårsages af *Salmonella enterica*, *Campylobacter jejuni* og *Campylobacter coli.* Disse bakterier spredes fra husdyr til mennesker via mange forskellige smitteveje inklusive fødevarer. Den udbredte brug af antibiotika i husdyrproduktionen og den deraf følgende risiko for udvikling og spredning af antibiotikaresistente bakterier er velbeskrevet i den videnskabelige litteratur. Sammenhængen mellem humant forbrug af antibiotika og potentielle konsekvenser for infektioner hos mennesker er derimod kun sparsomt dokumenteret.

Denne afhandling belyser sammenhængen mellem antibiotikaforbrug i mennesker og risikoen for infektion med antibiotikaresistente non-tyfoide *Salmonella, Campylobacter coli* (*C. coli*) og *Campylobacter jejuni* (*C. jejuni*). De primære formål var:

1) At vurdere om en tidligere eksponering for antibiotika hos mennesker er en risikofaktor for at blive inficeret med en antibiotikaresistent stamme af *Salmonella* eller *Campylobacter*.

2) At sammenligne symptomer og det kliniske forløb af sygdommen blandt patienter inficerede med *Salmonella* Typhimurium med forskellige antibiotikaresistensprofiler (i.e. fuldt følsomme, resistente eller multiresistente).

3) At undersøge om tidligere eksponering for antibiotika giver anledning til et alvorligere kliniske forløb af infektionen.

Kapitel 2 giver et generelt overblik over opdagelsen af antibiotika og mekanismer for udvikling af antibiotikaresistens i *Salmonella*, *C. jejuni* and *C. coli*. Den humane epidemiologi, kilder til infektion, antibiotikaresistensforhold, samt overvågning af *Salmonella* og *Campylobacter* i mennesker, dyr og fødevarer er beskrevet i hhv. kapitel 3 og 4.

Sammenhængen mellem et tidligere forbrug af antibiotika (dvs. antibiotika udskrevet pga. en anden diagnose) og risikoen for at blive inficeret med en *Salmonella* eller *Campylobacter* stamme, der er resistent overfor det samme antibiotika, beskrives i **Manuskript I** og **Manuskript II**. Begge studier var designet som registerbaserede case-kontrol undersøgelser. Data blev udtrukket fra flere forskellige nationale registre og sammenkørt via cpr. nummer. Omkring 10 kontrolpersoner pr. case blev matchet på køn, alder og bopæl. Tidligere forbrug af antibiotika blev trukket ud af Lægemiddelstatistikregistret; cases der indgik i studierne blev identificeret via Den danske mikrobiologidatabase (MiBa); sociale og demografiske forhold for cases og kontrolpersoner blev indhentet fra IDA-databasen (Integrated Database on Labour Market Research); og CPR-registret blev anvendt til at få oplysninger om alder og bopæl.

I alt blev der diagnosticeret 22.609 *Salmonella* tilfælde i Danmark mellem 1997 – 2005. Samtlige tilfælde blev inkluderet i studiet beskrevet i **Manuskript I**. Der blev fortaget separate statistiske analyser for hhv. *S.* Typhimurium (4.534 tilfælde), *S.* Enteritidis (4.195 tilfælde), og andre *Salmonella* serotyper (5.776 tilfælde). Vi fandt, at behandling med trimethoprim, sulfonamider, bredspektrede penicilliner, tetracykliner and fluorokinoloner, i op til et år før diagnose, kunne associeres med en forøget risiko for infektion med *Salmonella*. Den højeste risiko var forbundet med et tidligere forbrug af fluorokinoloner, og risikoen steg når tidsvinduet nærmede sig det estimerede infektionstidspunkt. Odds Ratioer (OR) for tidligere forbrug af fluorokinoloner var OR 4,6 (95% konfidensinterval (CI): 3,8 – 5,5) for andre *Salmonella*

serotyper, OR 2,2 (95%CI: 1,7 – 2,9) for *S.* Typhimurium, og OR 2,1 (95%CI: 1,8 – 2,4) for *S*. Enteritidis. Derudover fandt vi et interaktionsled mellem et tidligere forbrug af fluorokinoloner og det, at bakterien var resistent overfor fluorokinoloner; OR 3,6 (95%CI: 1,2 – 10,3) for *S.* Typhimurium og OR 2,7 (95%CI: 1,2 – 5,9). Det betyder, at risikoen for at blive diagnosticeret med en fluorokinolonresistent *S.* Typhimurium efter behandling med samme stof i op til et år før infektionen er 7,2 (2,0*3,6) gange højere for cases end for kontrolpersoner. For *S.* Enteritidis var den tilsvarende risiko 4,5 (1,7*2,7) gange højere. Disse fund tilskrives hhv. den kompetetive og den selektive effekt af antibiotikaresistensudvikling. Den kompetetive effekt opstår, når en antibiotikakur ødelægger den beskyttende effekt af den normale tarmflora. Den selektive effekt forekommer når en person eksponeres for et patogen, som er resistent overfor det antibiotika, som personen tidligere har indtaget. Sidstnævnte øger risikoen for infektion yderligere pga. det selektive pres som opstår på andre tilstedeværende bakterier, der er følsomme overfor stoffet og derved inaktiveres.

I perioden 1999 – 2005 blev der diagnosticeret i alt 31.699 tilfælde af *Campylobacter* i Danmark. Alle tilfælde blev inkluderet i studiet beskrevet i **Manuskript II**. Vi fandt, at det at blive diagnosticeret med campylobacteriose var forbundet med en forhøjet risiko for at have været eksponeret for fluorokinoloner, makrolider, bredspektrede penicilliner, tetracykliner, og sulfonamider og trimethoprim, i op til et år før infektionen. Risikoen var højest for fluorokinoloner (OR 2,4, 95%CI: 2,0 – 3,0). Da kun få *Campylobacter* isolater undersøges for resistens overfor andre antibiotika end fluorokinoloner og makrolider, var det kun muligt at beregne interaktionsleddet (i.e. kvantificere den selektive effekt) for disse to stoffer. I denne analyse fandt vi en yderligere effekt såfremt bakteriestammen tillige var resistent overfor fluorokinoloner (OR 1,6, 95%CI: 1,1 – 2,3). Odds for at blive eksponeret for fluorokinoloner var 2,4 gange højere for cases diagnosticeret med en fluorokinolon-følsom *Campylobacter* end for kontrolpersoner, mens odds for at blive eksponeret for fluorokinolon var 3,8 (2,4*1,6) gange højere for cases med en fluorokinolon-resistent *Campylobacter* infektion end for kontrolpersoner. For makrolider, var interaktionsleddet ikke signifikant (OR 1,0, 95%CI: 0,7 – 1,5). Cubic spline plots af OR for at blive eksponeret for makrolider, viste derimod en tilsyneladende beskyttende effekt af makrolidbehandling i op til en måned før tidspunktet for *Campylobacter* infektionen. Denne effekt kan forklares ved at såvel metabolitter som det aktive stof fanges i lysosomer på fagocytotiske celler, og derfra frigives langsomt, hvorved der opstår en forlænget virkning mod invasive bakterier som *Campylobacter*.

I **Manuscript III** undersøges sammenhængen mellem det kliniske forløb efter infektion med *S.* Typhimurium og resistensprofilen på den sygdomsfremkaldende Typhimuirum stamme, samt sammenhængen mellem klinisk forløb og tidligere antibiotikaforbrug. Det blev foretaget som en prospektiv case-case undersøgelse baseret på data fra telefoninterviews, som blev sammenkørt med data fra MiBa og CPR-registret. Data blev analyseret vha. logistisk regression. Interviewene blev foretaget i perioden januar-juni 2010, og i alt 150 *S.* Typhimurium cases blev inkluderet i undersøgelsen. Cases blev inddelt i tre grupper alt efter resistensmønstret på den Typhimurium stamme, som de var inficeret med: fuldt følsomme (S), resistente (R) overfor 1-3 antibiotika, eller multiresistente (MR), dvs. resistente overfor 4 eller flere antibiotika. Vi fandt, at et tidligere antibiotikaforbrug dvs. forbrug som ikke var relateret til *Salmonella* infektionen, gav en højere odds for vægttab (OR 2,4, 95%CI: 1,1 – 5,5), hospitalsindlæggelse (OR 2,0, 95%CI: 1,0 – 4,1), samt antibiotikabehandling for den pågældende *Salmonella* infektion (OR 7,9, 95%CI: 2,8 – 16,8). Undersøgelsen havde primært fokus på de kortsigtede kliniske konsekvenser (fx diarré og kvalme), da patienterne blev interviewet relativ kort tid efter deres diagnose blev registreret i MiBa. Dette forhold kan måske forklare, hvorfor vi, i modsætning til andre studier der typisk har haft fokus på langsigtede konsekvenser (fx dødelighed og sepsis), ikke kunne påvise flere alvorlige konsekvenser som følge af resistens. Det er også muligt at vi pga. studiedesignet ikke fik fat i de alvorligste tilfælde, simpelthen fordi de var for syge til at deltage.

Vi fandt også, at patienter inficeret med en resistent (R) *Salmonella* havde en højere odds for at blive hospitaliserede som følge af deres infektion (OR 2,5, 95%CI: 1,0 – 6.0), hyppigere havde mavesmerter (OR 2,9, 95%CI:1,3 – 6,5), og kvalme (OR 2,6, 95%CI: 1,1 – 6,2), end patienter inficerede med fuldt følsomme *Salmonella*. Vi observerede ingen stigning med stigende resistensniveau (S versus MR). Disse fund er måske også en afspejling af den kompetitive og selektive effekt af antibiotikabehandling (**Manuskript I og Manuskript II**), hvor tidligere behandling nedbryder eller ændrer sammensætningen af tarmfloraen i en grad der gør at infektionen får et alvorlige forløb. En alternativ forklaring kan være, at tidligere behandling er en indikator for en patient som er mere modtagelig for infektioner fx på grund af et nedsat immunforsvar.

Samlet konkluderes det på basis af resultaterne i denne afhandling, at humant forbrug af antibiotika påvirker risikoen for at blive inficeret med antibiotikaresistente stammer af *Salmonella* og *Campylobacter* på flere måder, og at behandling med antibiotika også kan være associeret med et alvorligere forløb af en infektion. Den tilsyneladende beskyttende effekt af makrolider, som observeret for *Campylobacter* infektion, tilføjer et yderligere lag til de komplekse sammenhænge. Mådeholdent forbrug af antibiotika bør altid anbefales – også til mennesker. Det anbefales at fremtidige studier fokuserer på at undersøge om sammenhængene fundet i denne afhandling kan genfindes for andre patogener.

List of abbreviations

CDC	Centres for Disease Control and Prevention
CFU	Colony forming units
DALY	Disability adjusted life year (an absolute measure of health loss)
DTU	Denmark's' Technical University (Danmarks Tekniske Universitet)
DVFA	Danish Veterinary and Food Administration
EFSA	European Food Safety Authority
GP	General Practitioner
ID50	The dose that will infect 50% of an experimental group, (infective dose for 50%)
MDR	Multi drug resistant
MLVA	Multiple locus variable number tandem repeat analysis
NHS	National Health Service
NREP	National Register for Enteric Patients
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
SSI	Statens Serum Institut
TESSy	The European Surveillance System
VNTR	Variable Number of Tandem Repeat analysis
95% CI	95% Confidence Interval

1 General Introduction

1.1 Introduction

Salmonella enterica, *Campylobacter jejuni* and *Campylobacter coli* are the most common causes of foodborne infections worldwide.[1] These pathogens are zoonotic, i.e., they can be transmitted from animals to humans, and a wide variety of animals have the potential of hosting these bacteria e.g. birds, cats, dogs, cows, etc. [2] Both bacterial species have many modes for transmission in the food chain through which humans can be infected. It is well acknowledged that the use of antimicrobial drugs in food animals leads to antimicrobial resistance in foodborne bacteria such as *Campylobacter* and *Salmonella.*[3, 4] However, the role of human antimicrobial use as a determinant for infections with zoonotic antimicrobial drug resistant bacteria is much less investigated.

This thesis focuses on two hypotheses relating to antimicrobial resistance and infection non-typhoidal *Salmonella, Campylobacter coli* (*C. coli*), and *Campylobacter jejuni* (*C. jejuni*):

1) Is previous antimicrobial drug use in humans associated with a higher risk of infection with non-typhoidal *Salmonella, C. coli* or *C. jejuni*?

2) Is infection with antimicrobial drug resistant *Salmonella* associated with more severe clinical outcome of disease?

1.2 Research Objectives

The main research objectives of this thesis were:

1) To assess if the history of human use of antimicrobial drugs is a risk factor for acquiring infection with an antimicrobial *Salmonella* or *Campylobacter* strain.

2) To compare clinical outcome of disease for patients infected with *Salmonella* Typhimurium with different antimicrobial susceptibility profile (i.e. pansusceptible, resistant or multiresistant).

3) To examine how clinical outcome of an infection is affected by previous antimicrobial exposure.

This thesis is divided into eight chapters. The introductory section, **Chapter 1**, is presenting the hypothesis and research objectives. **Chapter 2** describes mechanisms of antimicrobial resistance focusing on mechanisms found in *Salmonella* and *Campylobacter*. **Chapters 3 and 4** give a detailed description of the zoonotic pathogens *Salmonella* and *Campylobacter*, respectively. Each of these chapters describes characteristics of the pathogens, the disease that they cause, and surveillance systems in place in Denmark. The methodologies of the studies performed, and the data used are described in **Chapter 5**. The results, and an overall discussion are given in **Chapter 6**. Finally, **Chapter 7** provides a general conclusion and future perspectives.

2 Antimicrobial Drug Resistance

Antimicrobial treatment is the therapy of an infectious disease with an antimicrobial drug that either kills the pathogen or interferes with the growth of the pathogen. According to WHO antimicrobial resistance is: "Resistance of a microorganism (pathogen) to an antimicrobial medicine to which it was originally sensitive. Resistant organisms (including bacteria, fungi, viruses and some parasites) are able to withstand attack by antimicrobial medicines, such as antibiotics, so that standard treatment are ineffective and the infection persists and increasing the risk of spread to others. Resistance is a natural phenomenon, and certain resistant traits can be exchanged between certain types of bacteria. The misuse of antimicrobial medicines accelerates this natural phenomenon."

2.1 Discovery of antimicrobials and consequences of antimicrobial resistance.

History of penicillin, the first antibiotic and penicillin resistance

The first antibiotic drug that was marketed was penicillin, which was discovered by Alexander Fleming in 1928. In his article he concluded "that it had been demonstrated that a species of penicillium produces a very powerful antibacterial substance which affects different bacteria in different ways. Penicillin is the most effective against pyogenic cocci."[5]However, it took until 1939 before E. Chain managed to create a stable form of penicillin in the laboratory. In the US, in 1944, H. Florey designed a method to produce penicillin on a large scale. Only then, penicillin became widely used to treat wounded Allied soldiers during World War 2.[6] Not long after this discovery, for which Fleming, Chain and Florey received a Nobel prize in 1945, resistance against penicillin was discovered in 1945. However, resistance against this drug was discovered even before large scale production and use of the drug took place. In 1940, Chain and his colleague discovered an enzyme, penicillinase (β-lactamase), that could cleave the drug and inactivate it.[7] Once penicillin use became common, β-lactamase-producing bacteria became widely spread [6], due to selection pressure, which will be further discussed at the end of this chapter.

Antibiotic resistance has been on the rise since it was discovered in the first half of the 20th century, and it is a major concern for modern medicine since resistance rates in pathogenic organisms keeps rising.[8] Resistance to the first line of treatment is a problem since first line of treatment drugs are selected on the following grounds: safety, small spectrum and the lowest cost. Which makes second line of treatment drugs less desirable because they score worse on the abovementioned grounds.[9, 10] Multidrug resistance forms an even bigger problem because sometimes the second or third choice of treatment will not even be available. In the worst case scenario, doctors need to treat their patients with antibiotics that are toxic like colistin and fosfomycin.[11] Another problem with resistance against first line of treatment drugs is that these drugs are used for empirical, therapy, before antibiotic susceptibility results are available. Resistance against empirical treatment confers a worse outcome of disease because of a delay in effective treatment. Patients can be ill for a longer periods, have a higher mortality,[12, 13] and have higher odds to develop bloodstream infections.[14, 15]

Another side-effect of antimicrobial resistance is the economic effect, patients will be ill for a longer period of time, losing working days, spend longer time in hospital and use more and more expensive drugs, and other health care utilities.[16, 17]

2.2 Antibiotic resistance development

Besides development of antimicrobial resistance by bacteria, other factors play a role in the occurrence and the pace of this development. These factors are all influenced by human behaviour, and affect the selective pressure. Any use of antibiotics can increase selective pressure in a population of bacteria to allow the resistant bacteria to thrive and the susceptible bacteria to die off.[18-20] Human behavioural factors that play a role in the dissemination and spread of resistance are numerous and can be studied at a micro-level (the effect of antimicrobial therapy on the dissemination of resistance in the gut flora of a patient), or on a macro-level (for example; the effect of the amount of antimicrobials consumed in the veterinary sector on the occurrence of antimicrobial resistance in foodborne pathogens). Many books and articles describe these phenomena[21-24] , this chapter will only focus on a few mechanisms/theories that were crucial for the articles in this thesis.

Gastrointestinal flora (gut flora)

The gastrointestinal flora in the human gastrointestinal tract contains approximately 10^{12} CFU (colony forming units) per ml of colon-content, containing approximately 400 species of bacteria.[25] These bacteria help with the fermentation of non-digestible dietary residues, production of vitamin K , absorption of ions (Mg^{2+}, Ca^{2+}, Fe^{2+}, etc)[25], most importantly, these bacteria form a protection against pathogens (i.e. colonisation resistance).[26] Humans (and other mammals) live in symbiosis with their gut-flora: the host provides nutrients for the bacteria and the bacteria form a colonisation barrier in the gut. A problem arises as soon as the host consumes antimicrobials, these drugs are not only beneficial for the host to fight infection, but are detrimental for the gut flora, and thus for the host as well. Most antimicrobials taken will at some point pass the gastrointestinal tract, and cause part of the gut-flora to die off, this leaves the host vulnerable for opportunistic infections. Barza and Travers[27] described the competitive and the selective effect which was the foundation for the articles in this thesis.

Competitive effect

When exposing the intestinal gut flora to an antimicrobial, the intestinal flora will partly die off, due to this exposure. This will leave the patient with a low colonisation resistance and make him or her more vulnerable for weeks after the treatment, in which the patient has a higher likelihood to be colonised with any passing pathogen, until the natural gut flora is restored, which can take up to half a year after treatment with the antimicrobial. The competitive effect should apply equally to drug-susceptible and drug-resistant infections.

Selective effect

The selective effect occurs during the period that a course of antimicrobial treatment is taken. If, during this period, the person is infected with a drug-susceptible pathogen, the susceptible pathogen will be eliminated due to the antimicrobial. In case the infective pathogen is resistant to the antimicrobial taken, or the patient is asymptomatically infected with a pathogen resistant to this

antimicrobial, the pathogen will colonise the patient because the colonization resistance (i.e. the gut flora) is disrupted and will have space to proliferate.

The risk of developing antimicrobial resistance can be reduced by choosing antimicrobials with a minimal effect at the gut flora, i.e. narrow spectrum antimicrobials[28].

Use of antimicrobials in the veterinary sector

One of the main objects of this thesis is to address how much human antimicrobial use contributes to the occurrence of antimicrobial resistance in *Salmonella* and *C. coli* and *C. jejuni*. In 1969, the Swann committee advocated the prudent use of antimicrobials in the veterinary sector since the use of antimicrobials as growth promoters might lead to the dissemination of resistant organisms and their transmission to humans.[29] Ever since, it has been debated whether the main reason for antimicrobial resistance is use of antimicrobials in the veterinary sector or not. In 2005, the EU decided to put a ban on the use of antimicrobials as a growth promoter[30] as many studies demonstrated the association between the use of antimicrobials in the veterinary sector and occurrence of antimicrobial resistance in healthy animals.[31, 32] Other studies showed the bacteria isolated from human infections were the same as bacteria found in the animals, with the same antimicrobial resistance patterns.[33-36] Denmark was one of the first countries to put a ban on the use of antimicrobials as growth promoters in 1996 and studies have shown that this ban not only led to a lower prevalence of antimicrobial resistance in bacteria found in animals and animal products, but also led to a lower prevalence of resistance found in bacteria from human origin.[37] On the other hand, a clear one-on-one link cannot always be found, as in Australia fluoroquinolones have never been approved for use in the veterinary sector and resistance to fluoroquinolones is still found in *Campylobacter* isolates.[38] Since 2005, a list of antimicrobials that are considered critically important for human medicine exists, these antimicrobials are not supposed to be prescribed in veterinary medicine to preserve them for human use only.[39] Still antimicrobial resistance rates found in humans are increasing.[40] Suggesting that other factors, such as human antimicrobial use and misuse, and travel play a role in the dissemination of antimicrobial resistance.

2.3 Acquisition of antimicrobial resistance

Bacteria can be intrinsically resistant to antimicrobials (vertical transfer of resistance), acquire antimicrobial resistance through spontaneous mutations , or via the acquisition of resistance genes from other organisms (horizontal transfer). For horizontal transfer a three main mechanisms exist: transduction, conjugation, transformation.[41]

2.4 Management of bacterial diarrhoea and bacterial resistance mechanisms in *Salmonella* and *Campylobacter*

The most widespread mechanisms of resistance are enzymatic drug inactivation, active drug efflux, and reduced drug uptake. Other mechanisms are protection - and overproduction of the drug target, but these mechanisms are rare.[42] In this paragraph, only antimicrobial resistance mechanisms against clinically important antimicrobials against *Salmonella* and *Campylobacter* will be discussed. The cornerstone in the management of gastroenteritis remains rehydration, maintenance of hydration status and possible other supportive treatment. Antimicrobial drugs are usually not

recommended, but if the clinician decides to treat, the first line of drugs against *Campylobacter* will usually be a macrolide. Practically, patients are usually empirically treated with a fluoroquinolone. Since this is the first drug of choice to treat diarrhoeal disease when the causative pathogen is unknown. In case of bacteraemia due to *Campylobacter,* a course of aminoglycosides is administered intravenously. The first choice of treatment (if indicated) for *Salmonella* are fluoroquinolones, second- and third choice for treatment are trimethoprim-sulfamethoxazole (i.e. co-trimoxazole) and ampicillin. Children are usually not prescribed fluoroquinolones.

Fluoroquinolone resistance

The most common fluoroquinolone used to treat bacterial diarrhoea in humans is ciprofloxacin. Fluoroquinolones inhibit DNA replication by binding to either DNA gyrase (encoded by *gyrA* and *gyrB)* or topoisomerase IV(encoded by (*parC* and *parE*), and thereby disrupting DNA transcription and replication.[43, 44]

In both *Campylobacter* and *Salmonella,* fluoroquinolone resistance can be obtained through a mutation in a specific topoisomerase subdomaine named the quinolone resistance determining region (QRDR). Some mutations in this region lead to a decreased affinity of the drug to this region in the bacterium and thus increased resistance to fluoroquinolones. The rate in which these spontaneous point mutation in the DNA occurs is 10^{-6} to 10^{-9} cells in a large population.[45] Most amino acid changes occur in *gyrA* or in *parC*. One single mutation in *gyrA* can confer low level resistance, high levels of quinolone resistance are usually associated with a double mutation in the QRDR. Plasmid-mediated quinolone resistance, encoded by *qnr* -genes in both *Salmonella* and *Campylobacter* is another commonly found mechanism.[46, 47]

There has been ongoing debate on the MIC-values for quinolone resistance in Gram-negative bacteria for years[48-53]. Currently, the Clinical and Laboratory Standards Institute sets the susceptible MIC value at 0.06g/L for ciprofloxacin [54] and EUCAST set the same standard at $5*10^{-4}$ g/L for Enterobacteriaceae with a note that the MIC for *Salmonella typhi* is $6*10^{-6}$g/L and "The available data relate mainly to *S*. *typhi* but there are also case reports of poor response with other *Salmonellas*".[55]

Macrolide resistance

Macrolides (e.g. erythromycin, azithromycin, roxithromycin) bind to the ribosomes of the bacterium and hence interfere with protein synthesis.[56, 57] Macrolide resistance can be acquired through several mechanisms: 1) Modification of the ribosomal target by methylation of the 23S rRNA, in such a way that the macrolide cannot bind to it. 2) Efflux of the drug by the CmeABC pump, which is also used to pump fluoroquinolones out of the cell. 3) Modification of the antimicrobial by the activity of esterases and /or phosphotransferases.[44, 58, 59] Development of macrolide resistance has been observed in vivo, and can be acquired through point mutation.[60]

Trimethoprim-sulfamethoxazole resistance

This is a combination of two antimicrobials which are frequently given as a combination.[61] Both drugs work on the folic-acid synthesis pathway of the bacterium and have different mechanisms of resistance. Resistance to sulfamethoxazole (i.e. sulphonamide) is usually chromosomal. The mutation either leads to either an overproduction of a precursor of folic-acid (PABA), or to production of an altered enzyme (dihydropreroatesyntethase) on which the sulphonamide binds to block the folic-acid

pathway. Resistance to trimethoprim is acquired through either a mutation or by a plasmid which codes for an altered enzyme (dihydrofolatereductase) to which trimethoprim cannot bind.[62]

Ampicillin resistance

Ampicillin is a beta-lactam drug and resistance to this drug is often caused by production of beta-lactamases, of these TEM-1, SHV-1, PSE-1 and OXA-1 are the most frequently described mechanisms.[63] These beta-lactamases break down beta-lactam antibiotics, and can be transferred on plasmids.

Aminoglycoside resistance

Aminoglycoside resistance in *Campylobacter* is chromosomal, and the antibiotic is broken down by an enzyme. Except for kanamycin resistance, which is usually plasmid-borne. Resistance in this case is acquired by enzymatic alteration of the drug.[64] Kanamycin resistance is more common in *C. coli* than in *C. jejuni*, and is often found in combination with tetracycline resistance for which the resistance determinants are often found on the same plasmid.

3 Salmonella

3.1 Salmonella

Salmonella belong to the family *Enterobacteriaceae* together with other genera such as *Escherichia, Proteus* and *Enterobacter.* All these bacteria are characterised as Gram-negative, non-sporulating rods, facultative anaerobic, oxidase-negative (does not contain cytochrome c oxidase and can therefore not utilise oxygen for energy production), fermenting sugars to produce a variety of end products, e.g. lactic acid and acetic acid.[65] *Salmonella spp.* use flagella for their movement.

Nomenclature

Salmonella nomenclature is complex; there are more than 2600 known serotypes.[66] For a long time, two nomenclature systems based on a) Le Minor and Popoff[67] and b) the Bacteriological Code[68], were in use, which inconsistently divided the genus into species, subspecies, subgenera, groups, subgroups, and serotypes (serovars).[69]

Kauffmann and White proposed the first classification system in the 1920's and was based on serological classification of the O (somatic) and H (flagellar) antigens.[70] According to this system, each serotype originally was a separate species. In 1973, Crosa et al.[71] performed a polynucleotide sequence relatedness study. All serotypes and subgenera I, II and IV of *Salmonella* and all serotypes of *Arizona* were shown to be related at the species level and thus formed a single species *Salmonella cholerasuis*.[72] In 1986, the name *S. enterica* was proposed for the species *Salmonella*[73], as was previously proposed by Kauffmann and Edwards. This request for change in the nomenclature was, once more, proposed by LeMinor and Popoff of the WHO Collaborating Centre formally made a proposal as "request for an opinion" to the Judicial Commission of the International Committee of Systematic Bacteriology in 1987.[67] This request was denied by the Bacteriological Code which governs the taxonomy. Several researchers (Euzéby, Yabuuchi & Ezaki) have pleaded for an official statement to deal with the discrepancies between the nomenclature as proposed by Le Minor & Popoff and that which is officially recognised by the Bacteriological Code.[74, 75] In 2005 the Judicial Commission proposed a solution to the problem of nomenclature, even though they had no authority to make nomenclature changes that affect the taxonomy of the species.[76] Up to today the Bacteriological Code has not approved of any changes.

The nomenclature used in this PhD thesis is based on recommendations from the WHO collaborating labs, and was adopted by the National Reference Centre for Enteric Pathogens at the Statens Serum Institut (SSI) in Denmark and many other official institutions such as Institut Pasteur in Paris, France, which is the international WHO reference laboratory for *Salmonella*, and the US Centers for Disease Control and Prevention (CDC) in Atlanta, USA, is as follows: The genus *Salmonella* is split into two species: *S. bongori*, which has only one subspecies, and *S. enterica*, which is divided into six subspecies. Both species can be divided into the 2600 serovars mentioned earlier. In this thesis, *S. enterica* subspecies *enterica* serovar Typhimurium will be referred to as *S.* Typhimurium, and *S. enterica* subspecies *enterica* serovar Dublin as *S.* Dublin. Using phage typing, some serovars can be divided into phage types; *S.* Typhimurium DT104, *S.* Typhimurium U292, etc. Then, there is one more distinction to make, the difference between *Salmonella enterica* enterica serovar *typhi,*which causes

typhoid fever or enteric fever, and the non-typhoid serotypes. This thesis focuses only on infection with non-typhoid *Salmonella* species i.e. *S* Typhimurium.

3.2 Disease, Reservoir, Transmission and Epidemiology

Disease

Infection with *Salmonella* usually results in a self-limiting acute diarrhoea, lasting four to seven days. In some patients, episodes end in arthritis or other autoimmune complications, or the bacteria may spread and result in bacteraemia, meningitis or other invasive illness. In these cases antimicrobial treatment is necessary.[77] Most common and acute symptoms include, nausea, vomiting, stomach-cramps, diarrhoea, fever and headache. Several studies report on diseases that are directly linked to salmonellosis, especially post-infectious Irritable Bowel Syndrome (IBS).[78-80]

Reservoir

Salmonella can be found worldwide and has been isolated from virtually all vertebrate and invertebrate, and in the environment. It has always been thought that the main reservoir for human infection for non-typhoid salmonella is production animals.[81] A recent study of Mather et al.[82], may shed new light on this hypothesis though. By means of genome sequencing these researchers created a phylogenetic tree of 262 Scottish isolates of *S.* Typhimurium DT104, showing that circulation of DT104 occurred mainly separately in humans and in animals, with only a low frequency of spill-over in either direction.[82] Future research will have to proof whether this is valid for other phage types as well.

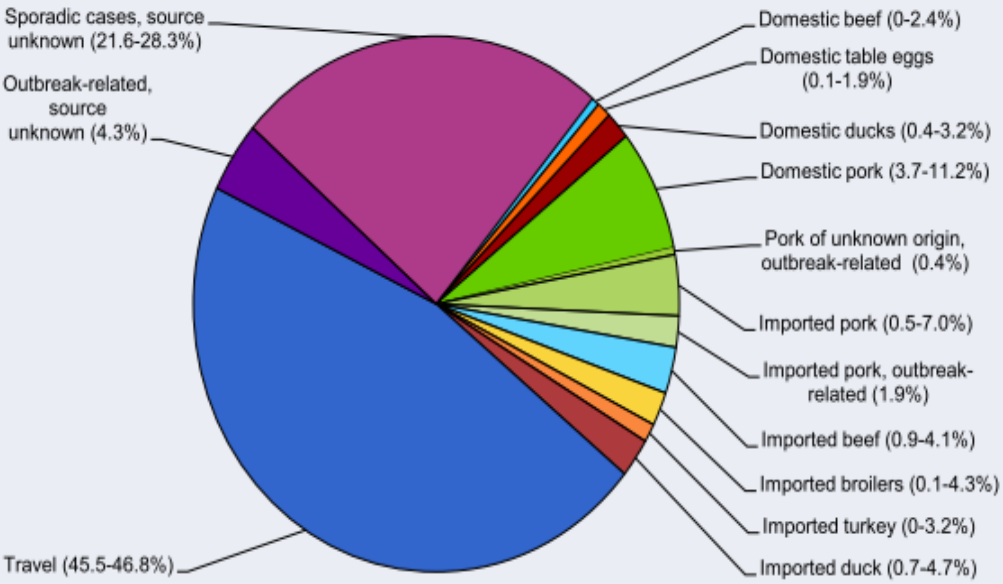
Transmission

Salmonella is transmitted through faecal-oral transmission, and usually has an incubation time between six and 48 hours. The infectious dose is relatively low; dose-response modelling of outbreak data suggests that an infective dose for 50% of the subjects (ID50) for infection is as low as 7 colony forming units (CFU) and the ID50 to cause illness was estimated at 36CFU's.[83] The ID50 is depending on the health status of the host and the vehicle in which the bacteria are suspended.

Four different ways of transmission for human infection can be distinguished; foodborne, contact with animals, environmental (including water) and person to person contact.[84-86]

Epidemiology

The most common source for human infections with non-typhoid *Salmonella* are foods of animal origin, typically meat and chicken eggs.[87]. These routes of food-borne transmission can theoretically be prevented by using proper kitchen hygiene and thoroughly cooking of the food products. Salmonellosis is more problematic when the bacteria are found in ready-to-eat products like instant milk for infants[88, 89], cheese[90, 91], chocolate[92], fresh herbs[93, 94], potato crisps[95], smoked salmon[96] and in ice-cream[97] to name a few. Over the past few years, fresh produce has become a more important source of contamination, which is on the increase, although imported pork still remains the most common source of infection, In Denmark.[98]



Source: Annual Report on Zoonoses in Denmark 2011
a) Sporadic and outbreak-related cases with unknown source include all sources not in the model. E.g. one outbreak with 43 cases where the source was tomatoes imported from Italy was included as "outbreak-related, source unknown"

Figure 3.1: Estimated sources of 1,166 cases of human salmonellosis, Denmark

Source: Annual report of zoonosis in Denmark 2011

Person-specific factors also play a role in the epidemiology of *Salmonella.* The incidence of non-typhoid *Salmonella* is usually the highest in young children and elderly people.[2] It should be noted that elderly patients often have a higher co-morbidity and higher 1-year mortality than younger *Salmonella* patients.[99] Behavioural factors such as less travelling, more institutional meals, or better kitchen hygiene amongst the elderly may counteract this surveillance bias.[100] Elderly patients are also more likely to develop serious consequences and would therefore require hospitalisation with a *Salmonella* infection. This is due to an impaired immune function, expressed in an elevated cytokine release by monocytes and neutrophils caused by an interaction of lipopolysaccherides with a serum factor in the blood of elderly.[101]

An elaborate study conducted in Denmark on risk factors for acquiring gastrointestinal disease identified a number of modest but noticeable associations:

Unlike what's noticed in third world countries, in Denmark the risk of several bacterial infections increased with increasing income level – this might be explained by differences in travel activity and dietary habits (eating out and eating more 'exotic' food).[84] Exotic food can be considered to be food that is not native to Denmark or Europe; ingredients that are used for Asian or African food for instance. Marked risk differences were seen for immigrants or persons whose parents were born outside Denmark. Specifically the risk of infection with bacteria associated with consumption of pork (*S.* Typhimurium) was markedly reduced.[102, 103]

Asymptomatic carriage of Salmonella

Asymptomatic carriage of Salmonella has been described[104-106], and carriers may harbour the bacterium in their liver and bile ducts, which is then excreted into the bowels . The most famous healthy carrier was “typhoid Mary”.[107]

As mentioned in a previous chapter on antimicrobial resistance, the human gut flora has several roles as a modifier in the risk of acquiring clinical salmonellosis upon exposure; first the gut flora provides a natural barrier for pathogens to cause damage (along with the gastric acid). On the other hand, the microbial intestinal flora can also form a niche for pathogens to hide and cause an opportunistic infection when this barrier of the commensal flora is washed out by a course of antimicrobials or other drugs. Healthy carrier status of people is well described feature of non-typhoid salmonellosis[108, 109], but there is one famous case found in history; typhoid Mary, figure 1. Mary Mallon was an Irish immigrant in the United States from 1863. This lady was an asymptomatic carrier of typhoid, at a certain moment she was known to have suffered a mild form of typhoid fever (*Salmonella* Typhi), and had recovered from it. Mary was working as a cook, and had to change households every time the family she had been cooking for, got ill with food-poisoning. By moving from family to family she infected 43 people of which three people died. After she was told by states health officers that she wasn’t allowed to cook anymore and was found cooking again, she was captured and held in a hospital where she eventually died 20 years later.

Figure 3.2: Typhoid Mary

Source: www.everseradio.com

Travers and Barza described the competitive and selective effect of antimicrobial resistance[110], see description in Chapter 2. The selective effect occurs during a period when an antimicrobial is taken, according to Travers and Barza, it is possible that a patient is colonised with an antimicrobial-drug resistant *Salmonella* in the past, but that the dose was too low to cause disease. In this case, it is likely that *Salmonella* can cause disease when the patient begins a course of antimicrobials, to which the bacterium is resistant too, causes selection and proliferation of the strain which can trigger an infection. This hypothesis is supported by the results from two studies described in this thesis;

Manuscript I and **Manuscript II**.[111, 112] This selective effect has also been described in cancer patients treated with chemotherapy and acquire *Salmonella* infection after, even though their stools were clean on hospital admittance.[113]

3.3 Burden

Incidence

Salmonellosis is an important public health issue worldwide. The global incidence of salmonellosis is estimated to be 93.8 million cases (95% CI: 61.8 – 131.6 million), with 155,000 deaths (95% CI: 39,000 – 303,000 deaths).[114] The reported incidence greatly differs between countries, and is dependent on the quality of the surveillance system, actual disease-load, health-care seeking behaviour of people, and laboratory practices. The European Food Safety Authority (EFSA) reports on salmonellosis in humans and food animals.[115] Human salmonellosis was reported 97,897 times in the member states of the European Union to The European Surveillance System (TESSy) in 2011[116]. Whilst Havelaar et. al. estimated the real incidence of *Salmonella* to be 6.2 million cases (95%CI 1.0-19 million),[117], this estimation was based on disease risk of a Swedish travellers study.[118] In this study, the risk of returning home after a holiday with campylobacteriosis and salmonellosis were calculated. They calculated that only one in every 58 cases of salmonellosis were actually notified in the EU.

The EU notification rate was 20.7 cases per 100.000 population, and showed a 5.4% decrease in cases compared to the previous year. Within the in EU Member States, great differences in notification rates exist, and ranges from 1.6 confirmed cases in Portugal to 80.7 confirmed cases per 100,000 population in the Czech Republic.[119] However, surveillance systems in the 27 EU member countries differ greatly, making the numbers hard to compare directly. In some countries, only hospitalised cases are reported, whereas in other countries, the surveillance system is set up to capture all cases that are tested in the medical microbiological laboratories such as in Denmark.[120] The highest notification rate for these cases was for age groups 0-14 years.

In Denmark the number of laboratory confirmed cases for 2011 was 1,166 (21 per 100,000 inhabitants),[121, 122] and in 2010, 1598 cases were reported, corresponding to 29 cases per 100,000 inhabitants.[123]

Economic costs and disease burden

The burden of illness due to diseases like *Salmonella* can not only be measured by morbidity and mortality, other major factors such as of hospitalisation, disability, long-term sequelae, and the economic costs can also be measured. These costs are hard to estimate, and differ between countries. The annual economic costs of *Salmonella* was estimated to be around $25.5 million (€18.2 million) annually for Denmark in a study performed by Wegener et. al.[124] An estimation of the costs was also made in the UK in 2003, where modellers calculated the impact of the illness and the cost of the resources used.[125] This was an extensive study, not only taking the costs of the National Health Service (NHS) into account i.e. hospital costs, GP costs, laboratory testing cost, etc, but also the estimated time off work were adjusted for gender and occupational grouping. On top of that, the cost of people who did get infected but did not present to themselves at the GP or hospital were

included. According to this study, the average costs per case of salmonellosis was £606 (€702).[126] In 2010, a similar study was performed in the UK, and in that study the costs increased to £1282 (€1484) and £993(€1150) per case of *S.* Typhimurium and *S.* Enteritidis, respectively.[127] The costs for each sequel of salmonellosis were estimated from a questionnaire filled out by patients. The substantial difference in amount of money per case can probably be attributed to the difference in the data used to calculate the cost; the last study did not include the costs of laboratory tests, sample collection and analysis. It is likely that the study of Roberts et *al.* is an underestimation of the real costs. In developed countries ,mortality due to salmonellosis is low, but the associated morbidity remains high.[128, 129]

Underreporting and under-ascertainment

Due to the way surveillance systems are set up, national registration databases only contain a fraction of the number of infections that occur in the general population. Not all exposure to infected food leads to an infection, and furthermore only a fraction of infections results in disease, of which only some of the symptomatic cases will seek medical care, see Figure 3.3. Health care seeking behaviour of individuals is influenced by several factors. Most important are a) occurrence of bloody diarrhoea, b) fear that the symptoms might be indicative of a serious disease, c) long duration of diarrhoea. Other factors have also been associated with healthcare seeking behaviour, such as being under 5 years or over 65 years of age, low household income and male gender.[130, 131] It is estimated that between 10-20% of people with acute gastrointestinal symptoms seek medical care,[130, 132]and of those people seeking medical care between 3-19% submit a stool sample for culture.[130, 132]

Reporting of national data is usually based on the numbers that are reported to the national surveillance labs, as can be seen in the surveillance pyramid below. This demonstrates the underestimation of real cases seen in the population.

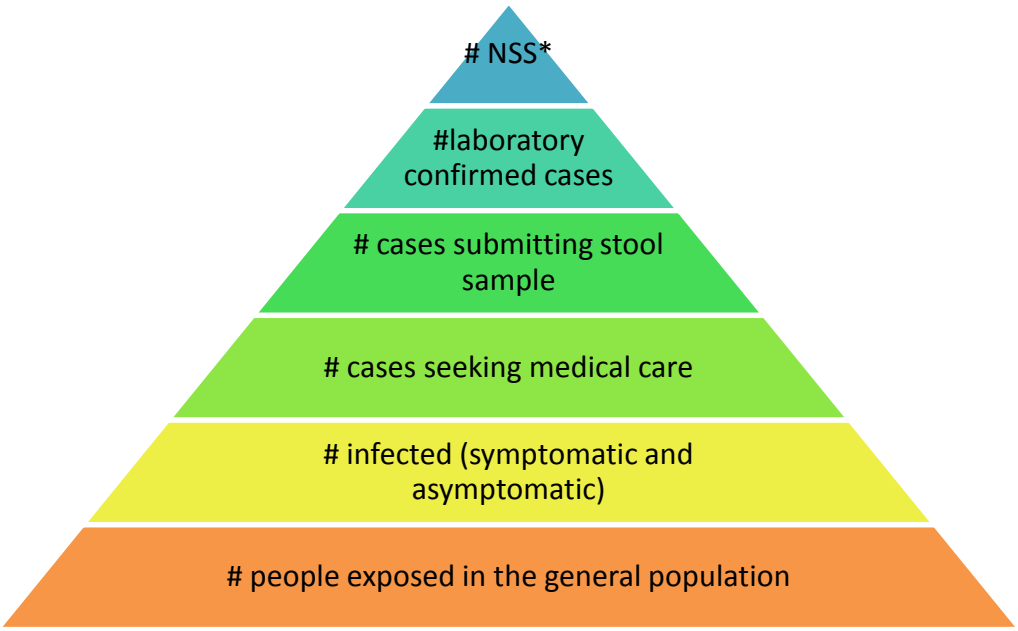


Figure 3.3; Pyramid of surveillance and underreporting of gastro-intestinal infections

***NSS: National Surveillance System**

Another approach to estimate the true incidence of *Salmonella* in various countries is sero-incidence estimation. This method has been developed for *Salmonella* by Simonsen[133]. This method uses the measurement of *Salmonella* serum antibodies in randomly collected blood sera to make an estimation of the sero-incidence in human populations from several European countries. This method led to an estimation of 325 infections per culture-confirmed case captured in the Danish National Surveillance Programme. The same approach has also been used on collections of blood-sera in other countries. Countries that were included in the study were; Finland, Sweden, Denmark, the Netherlands, Italy, Romania, France and Poland. Sero-incidences differed by ten-fold between the various countries, from 56 – 547 per 1000 person years. These estimates represented a ~100-2000-fold increase compared to the reported incidences by the National Surveillance Systems[134].

In conclusion, "serosurveillance" is a useful method to estimate the infection pressure within a country. This method is also useful for comparisons between countries, since the national surveillance systems in each country has a different set-up, and therefore a different sensitivity to pick up the true number of cases. On the other hand, serosurveillance is not a measure of the burden of illness, as an antibody response does not necessarily mean that the person got ill. It is merely an observation of the fact that a person came into contact with *Salmonella,* which elicited an immune response.

3.4 Typing

There are many different typing methods used to identify and characterise *Salmonella* for epidemiological investigations, surveillance and research purposes. The choice of method depends on the circumstances and the objectives, and often also the country in which the typing is performed. Typically, more than one method is used to improve the quality of the typing. In Denmark, all *Salmonella* isolates from human cases are serotyped.

Serotyping

Serotyping is the most basic and common form of *Salmonella* typing. Serotypes of *Salmonella* are based on the difference in the type of surface antigens(O) and the flagellar antigens (H). These antigens are detected using a slide agglutination test with commercially produced antisera. The O antigens uses a bacterial suspension from an agar plate, while H antigens uses a bacterial suspension from broth culture. The serotype is deduced from the specific pattern of agglutination reactions using the Kauffmann-White classification scheme. For instance *Salmonella enterica* serotype Typhimurium would have O antigens 4,5,12 , and H antigens i:1,2, the monophasic variant of *Salmonella* Typhimurium has O antigens 4,5,12 and H antigens i- (missing).

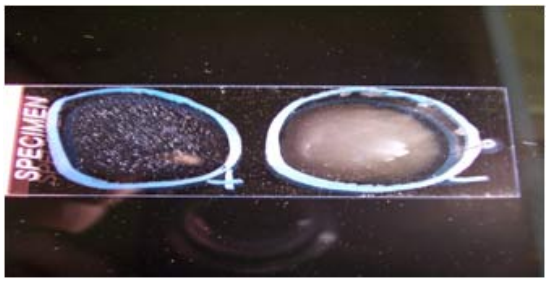


Fig 3.4: *Salmonella* serotyping with slide agglutination (Source: Food & Drug Admisnistration (FDA))

Phage typing

(Bacterio)Phage typing is a classical technique that is commonly used to subtype the more frequently occurring *Salmonella* serotypes like *S.* Typhimurium and *S*. Enteritidis. Phage typing is based on a set of specific bacteriophages that lyse the bacteria. Phages are viruses that can infect and destroy bacterial cells only, and are strain specific. The method is performed by spreading the bacterium on an agar plate, and drops containing the different phages are spotted. After overnight incubation, the plates are checked for plaques, which are clear zones that indicate a phage has inhibited the growth of the bacteria. The susceptibility of the strain to the different phages can be compared with known phage types.[135] It is likely that molecular typing methods will replace this phenotypically based method because it is difficult to maintain phage stocks and because of problems with standardization between labs.

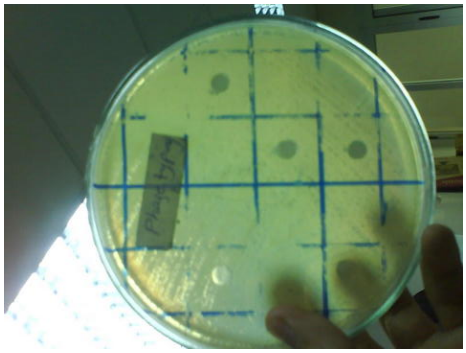


Figure 3.5: Example of *Salmonella* phage typing (Source: alexmedicalteam2014.blogspot.nl)

Pulsed Field Gel Electrophoresis (PFGE)

PFGE is a highly discriminatory method for differentiation of bacterial isolates on the basis of DNA content. This method uses specific restriction enzymes to cut the DNA producing fragments of different sizes. These fragments can range between 20-500Kbwhich are resolved using a specialized electrophoresis setup. The resultant banding patterns can be compared with one another strain patterns to determine genetic relatedness, see Figure 3.6 below. Because of the high discriminatory power of PFGE, it is frequently used in outbreak investigations. It should be noted that random genetic events, like point mutations or insertions or deletions as well horizontal transfer of mobile genetic elements e.g. plasmids can alter the banding patterns. Therefore it is recommended that at least ten distinct fragments of DNA are resolved for a comparison.[136] PFGE is currently a golden standard for subtyping. [137, 138]

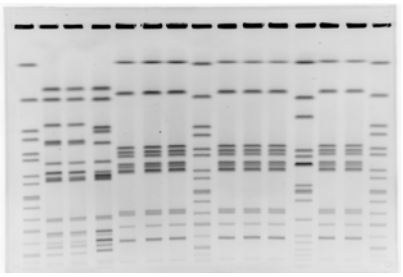


Fig 3.6: Example of *Salmonella* PFGE-patterns

Source: foodscience.cornell.edu

Multiple locus variable number tandem repeat analysis (MLVA)

MLVA has been used for typing *Salmonella*. It uses the naturally occurring variation in the number of tandem repeated DNA sequences found at specific loci within the genome. These loci are PCR amplified and depending on the band sizes, the number of repeats are estimated at each loci producing a combined string of integer numbers. At SSI (Denmark), MLVA-typing is commonly used for *S.* Typhimurium because it is more discriminatory then PFGE.[139]

3.5 Surveillance in Denmark

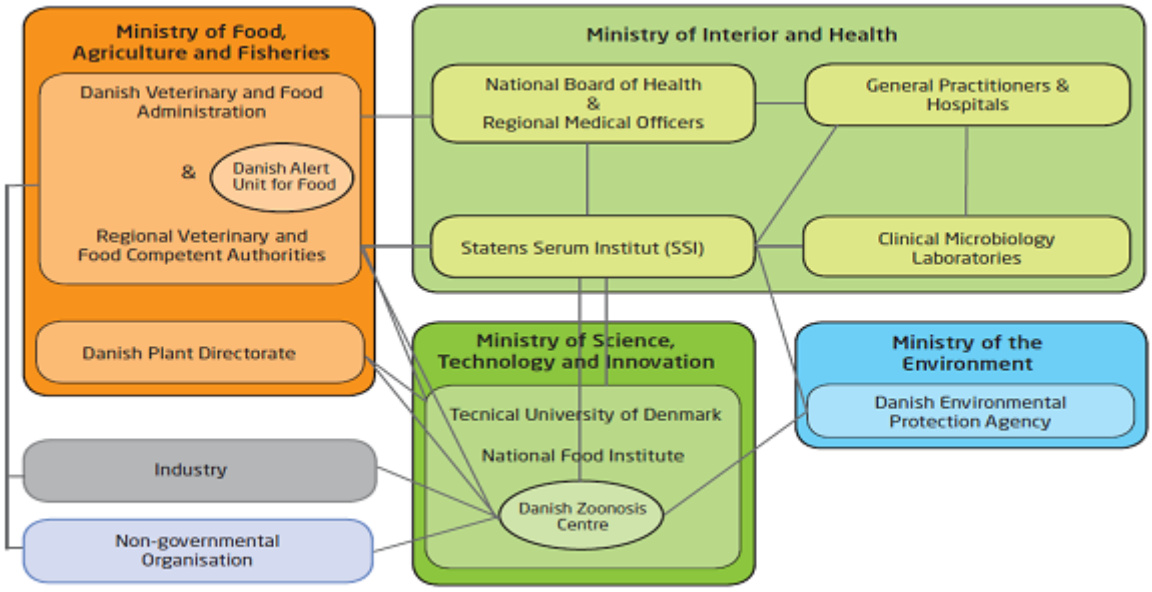
In Denmark the surveillance for *Salmonella* is performed in three different areas: humans, food producing animals, and food products.

Surveillance of Salmonella in humans

Diagnosis of human salmonellosis is performed by Enteric Reference laboratory at SSI and in 14 microbiology laboratories within the country. All laboratories must notify all *Salmonella* cases to SSI. All strains received at SSI are serotyped, antimicrobial testing is performed, and MLVA and/or PFGE are performed on selected isolates. MLVA is used for all cases of *S.* Typhimurium and has been shown to be useful for outbreak detection. Antimicrobial testing is performed using the diffusion method (Neo-Sensitabs, A/S Rosco, Roskilde, Denmark, using EUCAST clinical breakpoints). Phage typing was performed at the National Food Institute at the Technical University of Denmark, which is now outsourced to a private laboratory.

Since 2007, SSI also collects travel information on all *Salmonella* cases through a short interview performed by a special interview team at the Epidemiology Department. Information is gathered on whether patients had travelled abroad up to two weeks before onset of disease, and to which country they travelled to. This data is published in the DANMAP reports and in the Annual Report on Zoonoses.[98, 140]

Trends in human infections including clusters of cases are discussed at a weekly meeting at the Danish Zoonosis Centre, which is a network that includes the National Food Institute, SSI and the Danish Veterinary and Food Administration, see Figure 3.7 for a surveillance overview.



Source: Danish Zoonosis Centre, National Food Institute

FIG 3.7: OVERVIEW OF THE MONITORING AND OUTBREAK INVESTIGATION NETWORK FOR REPORTING INFECTIOUS PATHOGENS IN HUMANS, ANIMALS, FOODSTUFFS AND FEEDSTUFFS IN DENMARK, 2011

Surveillance of Salmonella in food producing animals

Poultry

Laying hens and broiler chickens in Denmark have a very low prevalence of *Salmonella* due to a top-down eradication programme.[141] The Danish National Surveillance programme for *Salmonella* has been in place since 1988 for broiler hens, and since 1998 for layer hens, and was reviewed in 2003.[142, 143] Infected flocks of breeding animals were removed from the breeding chain and slaughtered for consumption. As a result of this strategy, the number of *Salmonella* infected chickens and contaminated eggs in the Danish market was reduced drastically.[144] The incentive for farmers to co-operate with this programme was that they would receive a better price for meat that had a "Salmonella free-flock" label. Denmark also issued a ban on selling eggs from *Salmonella*- infected layer hens flocks, and now has now obtained a special guarantee for consumer eggs in the EU, meaning that Denmark can require that imported eggs to be *Salmonella*-free.

Pigs

Pig herds as well as pork from the slaughterhouses are routinely tested for *Salmonella.* Herds are categorised into three levels according to their sero-positivity. Owners of a *Salmonella* positive-herd are encouraged to seek advice on how to reduce *Salmonella*-infections on their farms using hygienic protocols, feeding schemes, etc. Furthermore, owners of sero-positive herds will receive less money from the slaughterhouse for their animals.[145] Due to introduction of the *Salmonella* control programme, the prevalence of *Salmonella* in pig herds has declined, but has not reached the same low levels observed in laying hens and broilers. [146]

Cattle

Since 2002, a *Salmonella* surveillance programme for cattle has been in place. This programme focuses on the eradication of specifically *S.* Dublin, since *S.* Dublin causes infection in cattle and is a major economical loss. *S*. Dublin can also cause severe disease in humans, sometimes leading to death[147]. However, due to the testing method (ELISA) used in the programme, cross reactions with other serotypes does occur. So the outcome of the surveillance, indicates the presence of most *Salmonella* species. Bulk tank milk samples are serologically tested, and the average of the last four test outcomes determines the category of contamination of the farm (three levels).[148] In an evaluation of the programme, performed by Ersbøll and Nielsen, using data between from 2003-2005, the incidence of positive herds declined from 22.1% to 17.0%.[149]

Surveillance of Salmonella in food products

From the late 1990s, imported meat was included in the *Salmonella* control programme, and from 2006, it was decided that all meat products on the Danish market should also be included. The Regional Veterinary and Food Competent Authorities samples imported batches of meat and meat of Danish origin for the presence of *Salmonella* and *Campylobacter.* This approach is called "case-by case-control". In this approach, meat is sampled from broiler, turkey, cattle and pork at the moment it is imported into Denmark.. For each batch, 12 pooled samples are taken and tested for *Salmonella.* If a sample is found positive, it is serotyped and tested for antimicrobial resistance[150].

3.6 Salmonella success clones

What is a "successful-*Salmonella* clone"?

Some *Salmonella* phage types seem to be more successful than other types in establishing an infection in a human host. To identify such subtypes that may emerge to be important in the future and to determine how these subtypes interact with host factors, we need to increase our knowledge on what makes a certain subtype "successful from the bacteria's point of view".

Success can be associated with several characteristics e.g. the ability to adapt to hostile environments, including acquisition of antimicrobial resistance (increased survivability), the ability to cause severe disease (degree of virulence) in humans and animals, and the ability to persist in the human or animal reservoir, or the environment for a long period. Table 3.1 includes examples of host factors and bacterial traits that may determine the success of a *Salmonella* subtypes.

The focus of this study was on the type of food source that might have led to the infection, the outcome of disease, some less conventional questions such as; food handling, and cleaning habits at home, and some general health questions on smoking, sports and drinking habits of the patients.

Table 3.1: Success clones: factors or bacteria-related traits that may make a *Salmonella* phage type more successful

Host factors	Underlying disease
	Medication
	Other factors compromising health
Virulence factor of the infective strain	Ability to acquire antimicrobial resistance
	Ability to cause severe disease
	Infection rate
	Ability to infect healthy hosts
Survivability in hostile environments	Freeze tolerance
	Resistance to toxic products
	Resistance to drying out

Successful clones

The following *Salmonella* Typhimurium phage types (or definitive types) were included in the study because they had one or more traits as described in Table 1.

DT104

The most well-known success clone is DT104. This phage type emerged worldwide during the 1990s[151-156]. This phage type is known to be multiresistant, usually being resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracyclines (i.e. resistant profile ACSSuT)[157]. In this phage type these antibiotic resistance genes have accumulated in chromosomally encoded gene cassettes. This process is mediated by the presence of class 1 integrons. Additionally, some isolates also possess plasmid-mediated resistance to trimethoprim and low-level resistance to ciprofloxacin, because of point mutations in the gyrA gene. This gene cassette has also been found in phage types DT12 and DT120, although DT12 is usually an antimicrobial susceptible phage type.

U292

In 2008 and 2009, a large outbreak (1466 cases) of *S.* Typhimurium U292 occurred in Denmark. Coinciding with this large outbreak, a smaller outbreak of DT135 (197 cases) and DT3 (89 cases) also occurred. These outbreaks were condined to Denmark and seemed to be epidemiologically linked, although a common source was never found. Outbreak investigation found that these outbreaks appeared to be linked to the consumption of traditional Danish food (e.g. pork).[158] Prior to the outbreak, all three phage types were uncommon in Denmark, but since the outbreak, a high proportion of the *Salmonella* Typhimurium cases in Denmark are due to U292 (36% in 2009[159], 11% in 2010[160], 8% in 2011[98] (since 2012 *S.* Typhimurium is not routinely phage typed anymore[161]). Both phage types DT3 and DT135, are rarely found in the past few years. In 2009, 5 and 16 domestically acquired cases respectively were notified, and in 2011, 3 cases of DT135 with a history of travel abroad were notified. All cases were domestically acquired. From this data it would appear that U292 has now successfully established itself in the Danish population. However, opposite other common *Salmonella* Typhimurium phage types, this type is rarely found in samples taken from livestock or food, and is fully susceptible to antimicrobials.

Monophasic

Since 2006, the monophasic *S.* Typhimurium 4,[5],12:i:− (hereafter monophasic *Salmonella*), emerged worldwide and has become one of the most frequently isolated *Salmonella* subtypes in many countries.[162-164] Monophasic *Salmonella,* are *Salmonella* strains that lack a second phase flagellar antigen. Strains missing the first phase flagellar antigen or both flagellar antigens are also found, but are less common. Monophasic variants are not separate serotypes or phage types, these types are mostly found in Typhimurium strains and are found to belong to several phage types. The most common type in Denmark is the monophasic DT193 variant[165].

The reason for the success of this type is still unknown. It could be that the gene *msgA* (which is macrophage survival protein), which is present in 4,[5],12:i-, makes the bacteria more likely to survive an attack on the immune system[166].

DT12 and DT120

Salmonella Typhimurium phage type DT12 was one of the most common *Salmonella* Typhimurium subtypes found in humans and animals, particularly slaughter pigs, during the 1990s. Here after, the occurrence of DT12 in both humans and slaughter pigs gradually declined [167], which may be associated with the fact that this subtype hasn't been very successful in acquiring antimicrobial resistance.[168] In contrast to DT12, DT120 has been increasingly found since 1994, and has also become more resistant to several antimicrobials over the past years, mainly due to the acquisition of a 43-kb genomic island called *Salmonella* genomic island I (SGI1). This pathogenic island makes it resistant to ampicillin, chloramphenicol, florfenicol, spectinomycin, streptomycin, sulphonamides, and tetracyclines. DT12 and DT120 are now the most common *Salmonella* types found in pigs and pork, and DT120 is the second most common type found in humans.[98]

3.7 Antimicrobial drug resistance in Salmonella

Global

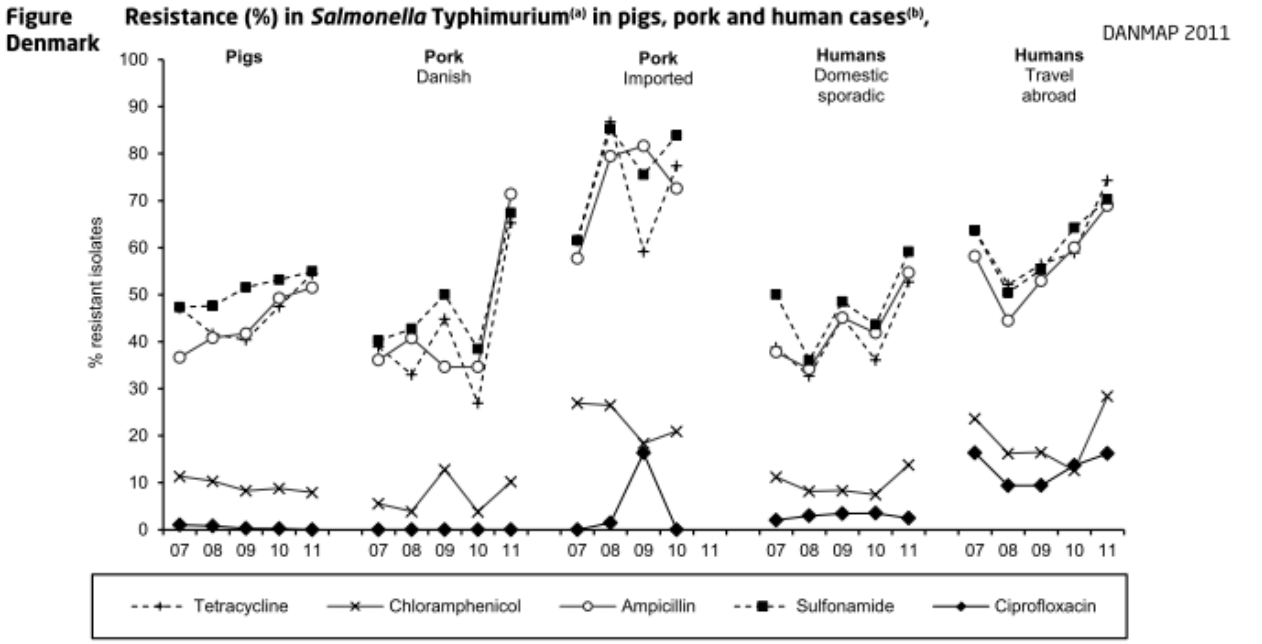
Since the publication of the Swann report in 1969, it has been recognised that antimicrobial use will lead to an increasing amount of antimicrobial resistance through selection pressure.[169] Antimicrobial resistance in *Salmonella* has been increasing worldwide over the past decades.[170-173] Of special concern is the rising resistance to first line treatment i.e. fluoroquinolones and third generation cephalosporins. This trend is especially high in South-East Asia and some African countries, where the highest morbidity for salmonellosis has been found.[174-180] In Vietnam, multidrug resistance is found in 50% of strains, and resistance to nalidixic acid (fluoroquinolone) was found in 97% of all 1,193 collected strains.[181]

In the EU

Salmonella resistance trends are gathered by EFSA. Overall, the most common *Salmonella* resistance profile belong to the ASSuT-profile (resistant to ampicillin, streptomycin, sulphonamides and tetracycline), which is associated with the most common phage types: *S.* Typhimurium DT193, DT120, and to a lesser extend DT7.[182]

In DK

Within Denmark, trends on antimicrobial resistance in *Salmonella* are gathered in the yearly DANMAP report. This report makes a distinction between travel-related cases and cases that are domestically acquired. The same difference is made for imported meat and Danish meat. This is done because of large differences in resistance found between the two sources for *Salmonella.* As can be seen in Figure 3.8, resistance against ciprofloxacin is alarmingly high(15-20%) in human cases that are acquired abroad. Guidelines for empiric treatment cut-off values for resistance of 5% are usually considered, meaning that empiric treatment with ciprofloxacin is not an option for this group of patients.[183] In 2011, ciprofloxacin resistance in *S.* Typhimurium were 2% and 16% in domestically acquired and travel-related cases, respectively. For *S.* Enteritidis, these 18% and 24%, respectively. Ciprofloxacin resistance in 2011 has increased 10% compared with previous years.[98] Resistance to cephalosporins was found in 1% of domestically acquired cases and in 12% of the travel-acquired cases.[98]



a) The number of isolates varies between years (pigs n = 202–581, Danish pork: n = 26–103, imported pork: n = 26–68, domestic sporadic human cases: n = 98–269 and travel related human cases: n = 55–117). Include isolates verified as monophasic variants of S. Typhimurium with antigenic formulas S. 4,[5],12:i:-. Data for imported pork in 2011 was not presented due to insufficient number of isolates
b) The isolate was categorised as 'domestically acquired' if the patient did not travel one week prior to the infection and as 'travel abroad reported' if the patient travelled one week prior to the infection

Figure 3.8 Resistance in *Salmonella* Typhimurium from different sources, Denmark, 2011.

Source: DANMAP 2011.

4 Campylobacter

4.1 Campylobacter general

The genus *Campylobacter* belong to the class of epsilon-proteobacteria. *Campylobacter* are Gram-negative, oxidase positive, curved rods, and belong to the same order as *Helicobacter*. *Campylobacter* are adapted to colonise the mucosal surface of the gastrointestinal tract. *Campylobacter* have long flagella, which in combination with their spiral shape, enables them to move rapidly in a "corkscrew"-motion through the mucous.[184, 185] For optimal growth, thermophilic *Campylobacter* such as *C. jejuni*, require a microaerobic environment and incubation temperatures of 37–42°C.[186]

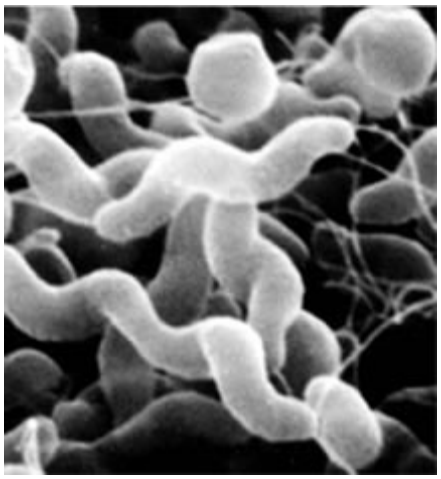


Figure 4.1: *Campylobacter spp.*

SOURCE: CAMPYLOBACTER.ORG

Nomenclature

Overall, there are 16 species and six subspecies of the genus *Campylobacter.* The two main species associated with human infections are C*. coli* and C. *jejuni*. Both *C. jejuni* and *C. coli* are thermophylic and are closely related, with the difference that *C. jejuni*, is capable of hydrolyzing sodium hippurate and *C. coli* cannot.[187] This thesis will only cover *C. jejuni* and *C. coli* infections, therefore, only these two species are meant with the term *Campylobacter* in the rest of this thesis

Clinical manifestation of *C. jejuni* and *C. coli* infection

After 2-5 days incubation, a *Campylobacter*-infection usually commences with sudden cramps and diarrhoea.[188] Other symptoms can include myalgia, nausea, bloody stools, headache, and vomiting.[189, 190] *Campylobacter* usually causes a self-limiting diarrhoeal disease but in some cases, campylobacteriosis can lead to an autoimmune disease called Guillan Barré Syndrome (GBS), which is a form of neuromuscular paralysis, and later onset of reactive arthritis.[191-194] Sepsis, and death due to campylobacteriosis are rare but can occur occasionally.[195, 196]

Reservoir and transmission

C. coli and *C. jejuni* are ubiquitous, and can be isolated from most mammalian species and birds.[187] *C. jejuni* is particularly suited to infect birds, since the body temperature of these animals is higher (41-42°C), which is an optimal temperature for *C. jejuni* to grow and proliferate.[197] The main reservoir for *C. coli* and *C. jejuni* is production animals and wildlife.[198-200] Transmission to humans has been described from contaminated water sources,[201, 202] consumption of contaminated meat, in particular fresh poultry[203, 204], raw poultry food products, and other raw animal products e.g. raw milk. Direct contact with pet animals has also been shown to be a mode of transmission.[204-207] Person-to-person spread is rare.[208]

Epidemiology

Between 85-95% of all *Campylobacter* cases in Europe are caused by *C. jejuni*, which may be the most common cause of bacterial food-borne diarrhoeal disease throughout the world.[209] In Denmark, more than 95% of the cases are caused by *C. jejuni*.[121] *C. jejuni* is most often isolated from broiler chickens, cattle and turkeys, whereas *C. coli* is predominately found in pigs.

Most cases of *C. coli* and *C. jejuni* infections occur sporadically, but they are known to cause large outbreaks, with a variety of sources implicated; poultry, raw milk, natural water pools, and drinking water.[210-217]

Campylobacter notifications within the EU show a seasonality with the highest incidences in the summer. It is hypothesised that is due to people eating raw poultry meat from BBQs [218], but high *Campylobacter* notifications are also correlated with environmental factors such as temperature, rainfall and agricultural density.[219-221] A number of *Campylobacter*-infections are acquired during foreign travel, and returning travellers during the summer may also contribute to the seasonality.[222]

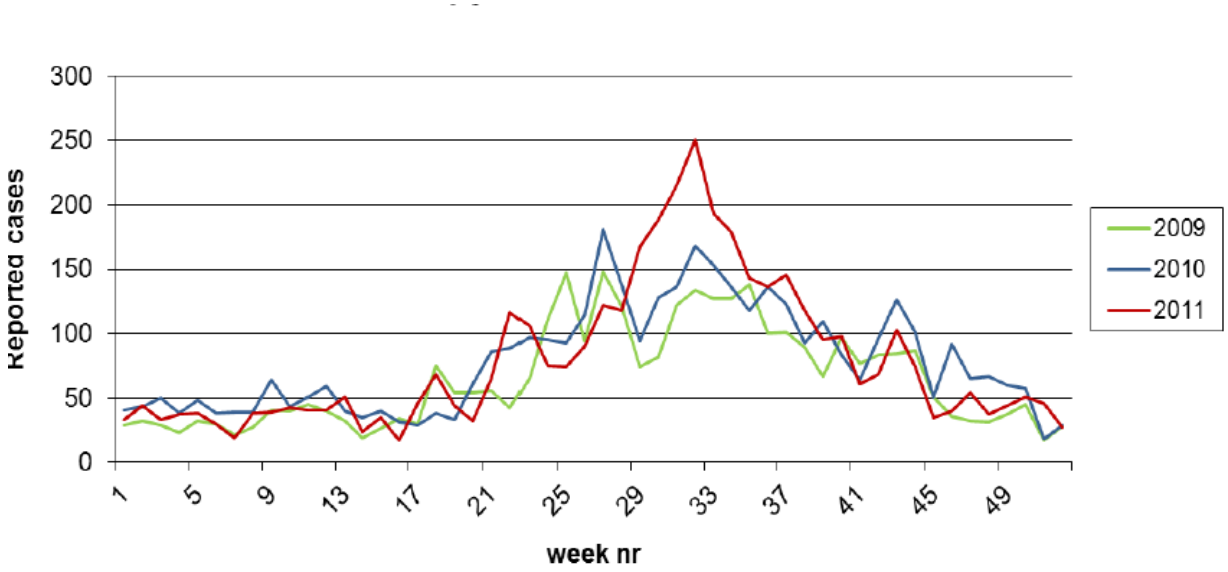


Figure 4.2: Seasonality of *Campylobacter* cases 2009-2011

Source: van Alphen, ESCAIDE 2012

The infective dose for campylobacteriosis is estimated to be low as 500-800 CFU, depending on host factors and the nature of which the *Campylobacter* was acquired.[223]Gastric acid generally is a good natural barrier for campylobacteriosis as *Campylobacter* are sensitive to the acidic-environment. Bouwknegt et al. (2013) found that consumption of proton-pump inhibitors which reduces the production of stomach acid, and is linked to a higher incidence of campylobacteriosis.[224]

Gut flora

In several animals, *Campylobacter* is commonly found without causing disease to the animal, especially in cattle, which are known to be asymptomatic carriers.[225] Humans have also been observed to be asymptomatic carriers.[226-228]

4.2 Burden

Incidence and economic costs

In 2010, the estimated global number of deaths due to campylobacteriosis was 109, 700 deaths (95%CI 81, 800–137, 200)[229]. The amount of disability adjusted life years (DALYs: an absolute measure for health loss) in that same year due to *Campylobacter* infections was 7,541/100,000 DALYs (95% CI 5,687–9,374).[230] The highest burden of disease for campylobacteriosis is in children under 5 years in both developing and developed countries.[231] The high disease burden in this age group is probably due to the fact that host immunity plays a large role in acquisition of a symptomatic *Campylobacter* infection.[231] According to Coker et al., the incidence of campylobacteriosis in children <5years of age differs in developing countries between 40,000 – 60,000/100,000 to 800/100,000 in the developed world.[232, 233]

In 2011, human campylobacteriosis was the most often reported zoonosis in the European Union with more than 220,000 cases.[234] The EU-notification rate was 50.3/100,000 population, and showed a 2.2% increase in comparison with data from the previous year. There is great differences in notification rates exist within EU Member States, ranging from 178 /100,000 inhabitants in the Czech Republic to 0.31/100,000 inhabitants in Latvia. As mentioned in the previous chapter 3, the surveillance systems in EU Member States differ. In some countries only hospitalisations are reported, while in other countries there are enhanced surveillance systems. Havelaar et. al. (2009) estimated the incidence of campylobacteriosis by using Swedish traveller data and found incidences between 30-13,500/100,000 population, with the highest incidence in Bulgaria, and the lowest incidences in Sweden and Finland. In Denmark it is estimated that there are 251/100,000 population[231], and in 2011 it was 73.1/100,000 population, which was similar to the incidence reported in 2010.[121]

The actual cost of gastrointestinal disease does not only encompass direct costs made by the patient visiting the GP, and the hospital, but also include indirect costs due to days of work lost because of illness. Direct costs may also include the health care costs due to reactive arthritis and GBS after a *Campylobacter* infection. In Denmark, no actual costs-of-illness studies have been performed, but Mangen et. al. (2005) performed a study in the Netherlands, which is a country similar to Denmark in terms of the health care system. The researchers calculated that the annual costs as a result of campylobacteriosis are approximately 21 million Euros per year. The Dutch population is

approximately three times the size of the Danish population, and the estimated incidence[117] in the Netherlands is approximately 6 times higher in Denmark; meaning the Danish annual cost would be approximately 1.2 million Euros per year, based on the costs calculated in 2005. However, this is a very rough calculation, and other cost-of-illness studies estimated 77[235]- 780 Euro[236] per case of gastroenteritis, depending on the variables are included in the calculation, both studies conclude that indirect costs (productivity loss) due to illness make up the largest sum.

Underreporting and underascertainment

Like salmonellosis and other gastrointestinal diseases, campylobacteriosis is underreported[237]. Not everyone who suffers from acute gastroenteritis will visit a GP, and not all of the cases that are reported to the GP will provide a stool-sample for analysis, see Figure 3.3 on page 20 in Chapter 3. Seroprevalence studies may be applied to estimate the exposure to *Campylobacter*. This is a method which uses serum antibodies as a biomarker to estimate seroconversion rates as a proxy for infection pressure. This method, performed by Teunis et. al. estimated a rate of 0.80 infections per person per year.[238]

4.3 Typing

For general diagnostic purposes, *Campylobacter* are usually not identified to the species level, e.g. no difference is made between *C. jejuni* and *C. coli*. However, for surveillance purposes, identification of to the species level is usually performed in a subset of strains. Further strain typing is usually not carried out as a routine activity using phenotypic methods like serotyping and phage typing.[239] Various genetic methods that can be used for typing include a species specific PCR assay on restriction fragment length polymorphisms (RFLP), *fla*-typing, PFGE and ribotyping.[240-242]

4.4 Surveillance in Denmark

Surveillance of *Campylobacter* in humans

Since 1980, culture based national surveillance for *Campylobacter* has been carried out in Denmark.[243] *Campylobacter* is a notifiable disease and all laboratory confirmed human cases are reported through the National laboratory surveillance system to the National Gastrointestinal Unit at SSI and entered into the National Register of Enteric Pathogens.

Surveillance of *Campylobacter* in food producing animals

All broiler flocks are under surveillance for *Campylobacter* since 1998. Before 1998, a subset of all flocks was monitored for DANMAP. Since 1995, pigs and in cattle (caecal content of one animal per herd) were also included in the monitoring scheme. Only on suspicion, are wildlife, zoo-animals and pets screened for *Campylobacter*. These numbers are low and not representative for surveillance purposes but do get published in the Annual Report on Food Zoonosis every year.

Surveillance of *Campylobacter* in food products

The Danish Veterinary and Food Administration (DVFA) collects samples from meat sold at wholesale and retail outlets. The *Campylobacter* species are identified and antimicrobial testing is performed at

the National Food Institute. For *Campylobacter*, the case-by-case-control is also applied, see chapter 3.

4.5 Antimicrobial drug resistance in *Campylobacter*

Global

Antimicrobial resistance levels in *Campylobacter*, in particular to macrolides and fluoroquinolones are of concern since these two drugs are the first line treatments for severe infections with this bacterium. Resistance against both drugs have been emerging globally in the past decades.[244-246] An example is erythromycin (macrolides) resistance, which is mainly found in strains of animal origin especially *C. coli* originating from pigs and chickens.[247]

EU

Antimicrobial resistance in EU Member States is monitored by both EFSA and ECDC, and the yearly results are published in The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food. In the latest report on the data from 2010, it was reported that resistance levels of *Campylobacter* from human isolates against fluoroquinolones (~45%), ampicillin (~35%) and tetracylines (~30%) were high, whilst resistance levels to macrolides (~15%), remain at a comparable low level in the EU.[234] In *Campylobacter* from animal origin i.e. fowl, pigs, cattle and broiler meat, levels of resistance against fluoroquinolones and tetracycline were high, whilst resistance levels against macrolides and aminoglycosides were low.[234] A variety of testing methods are used in the different countries, both disk diffusion and dilution methods, and a different guidelines to interprete resistance. Laboratories use either CLSI, EUCAST, British Society for Antimicrobial Chemotherapy or the French Society for Microbiology guidelines. Interpretation differences of one or even two dilution steps exist between the guidelines. Although, according to EFSA and ECDC, laboratories and societies like CLSI and EUCAST, are working to diminish the differences that exist between these guidelines.[234]

DK

Denmark collects data on resistance for *Campylobacter* isolates of human origins in two categories: "domestically acquired" and "travel-related". The levels of antimicrobial resistance differs greatly between the two categories. Since 2007, SSI collects data on travel history of patients in the National Registry for Enteric Patients. Patients are asked about the date of onset of disease and whether they had travelled abroad in seven days prior to onset of disease. If the patient had not travelled abroad then the infection is categorised as "domestically acquired". Resistance to fluoroquinolones in domestically acquired cases are 33% and in travel-related cases it is 84%, Resistance to macrolides are 0% and 3% respectively.[121]

5 Materials & Methods

This chapter describes the materials and methods used in the studies included in this thesis. The chapter consists of three sections: 1) a description of the databases used extracting data for all three studies, 2) a description of the original and final set up of the telephone-interview study (**Manuscript III**), including data collection and analyses, and 3) the data and methods applied in **Manuscript I** and **Manuscript II**.

5.1 Databases in Denmark

CPR registry (Civil Registration System)

Since 1968, upon birth or immigration, every Danish citizen and resident receives a CPR number, which is a unique personal identifier. All CPR-numbers are kept in the Danish Civil Registration System; within this system CPR-numbers are associated with the name, gender, civil status, citizenship, date of birth, and vital status of each individual.[248-250]

For the studies included in this thesis, encrypted CPR-numbers were used to link data from different national registries. All patient data was held and linked by Denmark Statistics on whose servers the analyses of the first two manuscripts were performed.

National Registry of Enteric Pathogens

This registry contains data on all infections with gastrointestinal bacterial pathogens, confirmed by faecal culture or by culture from a normally sterile site are diagnosed at either SSI or one of ten local clinical microbiological laboratories. In cases where a patient tests positive for the same bacterium (e.g., *Salmonella* serotype or *Campylobacter* spp.) multiple times within six months of the first diagnosis: these cases are considered to be recurrent or persistent and consequently, the laboratory enters only the first episode into the database. Each entry is coupled to the patient's CPR number, date of onset, pathogen and antimicrobial susceptibility profile of the pathogen.[121, 251]

This database was used in all three manuscripts to identify patients with either *Salmonella* or *Campylobacter* (*C. coli* and *C. jejuni*) infections.

Danish National Prescription Registry

Antimicrobial drugs are available by prescription only in Denmark, consequently their use can be monitored through the Danish National Prescription Registry (DNPR); a database available to researchers from Statistics Denmark, the data encompasses all prescriptions of drugs sold at pharmacies in Denmark since 1994. In 2012, the Danish Medicines Agency became the owner of the data, which is currently maintained at SSI. This registry contains information on dispensed prescriptions and includes data on the drug user (i.e. CPR number, gender, municipality and region of residence), the pharmacy (i.e. name and address), and details on the drug dispensed (i.e. ATC-code, dose, dispensing date, etc.). The DNPR does not contain data on over-the-counter drugs (drugs sold without a prescription like paracetamol), drugs administered to patients in the hospital, and the indication for prescribing the drugs.

Data from the DNPR was used to derive data on history of antimicrobial use in **Manuscript I** and **Manuscript II**.

Integrated Database on Labour Market Research

The Danish Integrated Database for Labour Market Research (IDA) has been developed and managed by Statistics Denmark since 1988 and is primarily used for research purposes. The registry contains information on socio-demographics (i.e. income, employment, gender, age, educational level, family and household).[252, 253]

Data from this database was used in **Manuscript I** and **Manuscript II** to control for the potential confounding effect of educational level and socioeconomic status.

5.2 The registry-based studies (Manuscript I and II)

Antimicrobial susceptibility testing on *Salmonella* and *Campylobacter*

Susceptibility testing of *Salmonella* and *Campylobacter* was performed on a sample of submitted strains received by the Unit of Gastrointestinal Infection of SSI. Susceptibility testing of *Campylobacter* against macrolides and fluoroquinolones was performed on a large percentage of strains, as these antimicrobials are first line of treatment drugs, testing against a more extensive panel of antimicrobial drugs was performed in a random sample of.

Antimicrobial drug susceptibility was tested by determining the minimum inhibitory concentration (MIC) with micro broth dilution method using a commercially available MIC technique (Sensititre, Trek Diagnostic System, UK), according to CLSI guidelines (Clinical and Laboratory Standards Institute) for samples before 2007. From 2007 on, EUCAST epidemiologic cut-off values (ECOFFs) were used. The cut-off values differed over the years included in the study (see DANMAP 1997-DANMAP 2005, and DANMAP 2010; www.danmap.org), for the analyses we included the cut-off points that were valid for the year each sample was entered in the NREP. The MICs were interpreted and denoted as S (susceptible), I (intermediately resistant), and R (resistant). All intermediate resistant samples were considered to be susceptible in all three manuscripts included in this thesis. Antimicrobial drug resistance tested for, and included in **Manuscript I and II**, are described in Table 5.1 below.

Table 5.1: Definition of the groups of antimicrobials included in Manuscript I and II

Groups of antimicrobials	ATC code	Antimicrobials tested for in susceptibility test
Aminoglycosides	J01GB	streptomycin, gentamicin, apramycin kanamycin, spectinomycin
Amphenicols	J01B	Chloramphenicol
Extended spectrum penicillins	J01CA	ampicillin, mecillinam
Fluoroquinolones	J01M	ciprofloxacin, nalidixic acid
Other antibacterials	J01X	collistin, nitrofurantoin, polymixin, fosfomycin,
Sulphonamids and trimethoprim	J01E	sulfamethoxazole, trimethoprim
Tetracyclines	J01A	Tetracycline
Third generation cephalosporins	J01DD	Ceftriaxone

Data

Salmonella

Data for **Manuscript I**, included samples from 1997 to 2005. In total, 97% (4,534/ 4,675) of *S*. Typhimurium, 35% (4,195/12,195) of *S.* Enteritidis, and 29% (1679/5776) of other *Salmonella* were susceptibility tested. Susceptibility testing for the following groups of antimicrobials were included in the study: aminoglycosides, amphenicols, extended-spectrum penicillins, fluoroquinolones, other antibacterials (ATC code J01X), sulphonamides and trimethoprim, tetracyclines and third generation cephalosporins. See Table 5.1 for the ATC-codes, and antimicrobials included in each group.

Campylobacter

Manuscript II included samples taken between 1999 and 2005. Susceptibility testing for macrolides and fluoroquinolones was performed on 10,275 (32%) of 31,669 patients included in the study. Resistance to other antimicrobials was performed on a much smaller percentage of the sample and was therefore not included in our analysis.

Methods

For **Manuscript I** and **Manuscript II**, similar methods were applied to calculate the odds that previous antimicrobial use has a long term effect on acquiring an antimicrobial resistant infection with *Salmonella or Campylobacter (C. coli and C. jejuni*). Descriptive analysis were given on the prevalence of resistance and the use of different classes of antimicrobial drugs, and conditional logistic regression was performed to investigate the odds of exposure to a course of antimicrobial drugs before diagnosis with either *Salmonella* or *Campylobacter*, and to calculate the odds of a strain being resistant to the antimicrobial previous taken.

Study design

Both **Manuscript I** and **Manuscript II** are registry based, matched case-control studies, conditional logistic regression was used to analyse the data.[254]

In these analyses, the odds that a person was exposed to an antimicrobial drug at six different time-windows before being infected with *Salmonella* or *Campylobacter*, and the odds that the infective strain was resistant against the antimicrobial taken were estimated. The time-line in Figure 5.1 shows how the data was divided into different time-frames prior to infection for the analysis of the data.

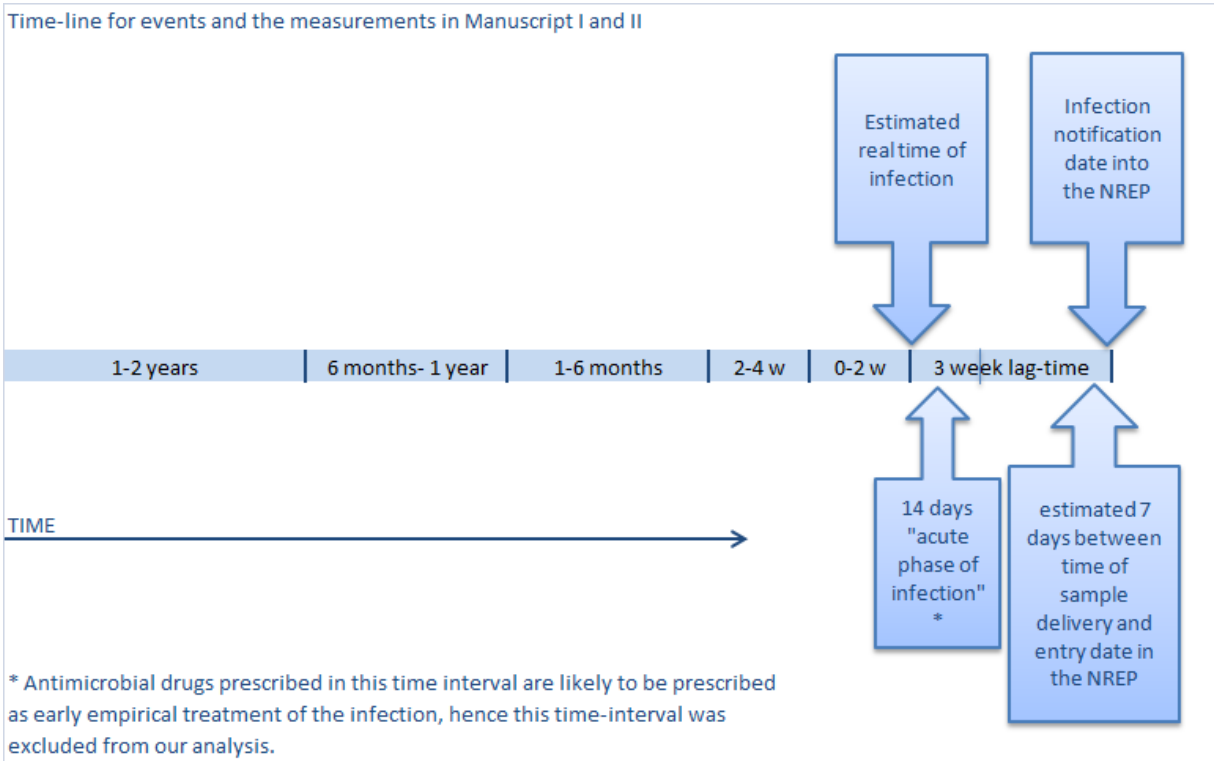


Figure 5.1 Time line for events and measurements in Manuscript I and II

The date of infection was derived from the NREP, and we deducted 21 days to estimate the real date of infection. Firstly, seven days are deducted, as we estimated that this would be the delay between the date that the stool-sample was handed in and the date that the infection was notified in the NREP. Secondly, we deducted another 14 days, which we called the acute phase of infection. We estimated that this would be the duration between infection-date and the date that a patient would hand in a stool-sample. We excluded this time frame from our analyses as we expected that any course of antimicrobials taken during this period might have been given as early empirical treatment of the infection. Including these data would introduce protopathic bias.[255, 256]

All *Salmonella* patients (**Manuscript** I) between 1997-2005 and *Campylobacter* patients between 1999-2005 (**Manuscript II**), entered in the NREP were included in each study. For each case, ten controls were selected that were alive on the date that the patient was entered in the NREP, and matched on age, sex, and county of residence. We adjusted for education level (primary school only or higher level than primary school), rural/urban differences by population density (separated into five categories; >2000, 1001-2000, 351-1000, 26-350, and 1-15 persons/km^2), and income (income per household / number of adults, and matched within a range of 100.000 Danish Kroner intervals.[257] Effect modification by age was determined by fitting an interaction term by three age groups (0-15 years, 15-64 years and ≥65 years).

The analysis consisted of two separate parts; firstly, overall odds ratios for exposure to antimicrobial drugs prior to the estimated onset of infection (index date) were calculated, and secondly, a time-dependent OR for exposure to antimicrobials was calculated, for each of the six time-frames, see Figure 5.1. The outcomes of these time-dependent calculations were plotted and cubic splines were applied to obtain a smooth curve.[258] The coefficients for the cubic spline function were estimated

in a conditional logistic regression model. In the cubic spline model three knot points were used; at 60 days, half a year and one year before the index-date. A knot point is a point where the coefficients for the third-degree polynomial were allowed to change, although only under the restriction that the curve has to be continuous and smooth at the knot points.

All analyses were performed using conditional logistic regression with the PHREG procedure in SAS 9.1.3 for UNIX (SAS Institute, Cary, NC, USA).

5.3 Prospective case-control study (Manuscript III)

Introduction

Part of this PhD-study was funded by the Danish Ministry of Food Agriculture and Fishery, through a large project entitled "Targeted approaches to the control of virulent and antibiotic-resistant *Salmonella* clones" (project-number 3304-FVFP-07-721-01). The telephone interview study was performed to meet one of the goals of this project: to describe outcomes of infection with specific "successful-clones", and find risk factors for acquiring an infection with a specific "successful-clone".

Successful-clones

As described in chapter 3 in the paragraph on successful clones, not every *Salmonella* subtype is as successful in establishing infection in humans, causing severe disease or in acquiring antimicrobial resistance. For the purpose of this study we focussed on these "successful" *Salmonella* Typhimurium subtypes only.

Original set up of the "telephone-interview study"

The original research objectives of this study were to identify specific risk factors (other than those that are food-related), for each of the above-mentioned phage types. Data were gathered by means of a telephone interview conducted by professional interviewers trained at SSI. Cases were interviewed about symptoms of infection, previous drug prescriptions, antimicrobial resistance pattern, general health status, food exposure, cleaning habits, smoking-status, etc.. In order to reduce recollection bias, the interviews were conducted as soon as possible after notification in the National Registry of Enteric Pathogens (NREP). The preferred study type was to perform a case-case study for the following reasons: Firstly, by comparing the phage types of interest with other *S.* Typhimurium cases, we would be able to identify the risk factors that are specifically associated only with the phage type under investigation. As both case-case patient and case-control would have the same aetiology, this would avoid introducing the general effect of being infected with a *S.* Typhimurium [259]. Secondly, the risk of selection-bias would be reduced, as both case-patient and case-control would be enrolled through the same surveillance system[260] Finally, we expected the willingness to participate among patients to be higher than the willingness of healthy controls of the general population, meaning that healthy controls, that are willing to participate may not be representative of their corresponding case.

Methods

Patients with a culture-confirmed infection with *Salmonella* Typhimurium and recorded in the NREP between January-June 2010 were eligible for enrolment in the study. Cases that belonged to an

outbreak, were infected with a strain that could not be phage typed or cases that had a history of travel within two weeks of onset of disease were excluded from the study. Only the first case of a household within a time-frame of a month was included in the study. The address and, age of the patients were derived from the CPR-registry, after which the telephone numbers were looked up on the internet. After this, the interviews were performed by the trained interview-team at SSI. Data was entered in a database during the interview by means of a market research platform, Defgo.net®.

Sample size calculation

A sample size calculation was performed to determine the amount of case-patients and case-controls that had to be enrolled in the study. We calculated that in order to show an OR of 3 with 80% power and 95% confidence interval, we had to interview 50 case patients of each phage type and 100 controls. These controls would be patients in the analyses of another phage type. In previous years, 1392 (in 2008), and 559 (in 2009), domestically acquired *S.* Typhimurium cases were entered in the NREP.[261] Based on these numbers we expected to interview enough cases for the study sample size.

Data

In total, 326 *S.* Typhimurium patients were recorded in the NREP in the period between January-June 2010, of which 228 were eligible for the study. After drop-out, 150 patients were interviewed. Reasons for drop-out are presented in the flow diagram, Figure 5.2. Isolates from these patients included monophasic *S.* Typhimurium (N=23) and four phage types of interest for the case-case study : DT104 (N=7), DT12 (N=8), DT120 (N=2), and U292 (N=17). According to our sample-size calculation not any of these subgroups was large enough to perform our analysis with enough strength, and due to time-limitation we were not able to prolong the study to get more patients enrolled. Therefore, the original aim of comparing risk factors for different subtypes could not be met. However, the objective of the project was to investigate the association between antimicrobial resistance pattern in relation with phage type and outcome of disease. Although we did not have enough cases in each group to look at phage type in relation to resistance, analysing the resistance-pattern in relation to clinical outcome of disease was possible. This, consequently, became the main focus of the study (**Manuscript III)**. Cases were classified into three groups according to the susceptibility profile of their infection: pansusceptible (S), resistant to three or less antimicrobials (R), or multiresistant (MR) i.e. resistant to four or more antimicrobials. The antimicrobial resistance testing panel contained data on resistance against 16 antimicrobial drugs: ampicillin, apramycin, cefotaxime, ceftiofur, chloramphenicol, ciprofloxacin, collistin, florfenicol, gentamicin, nalidixic acid, neomycin, spectinomycin, streptomycin, sulphonamide and trimethoprim .

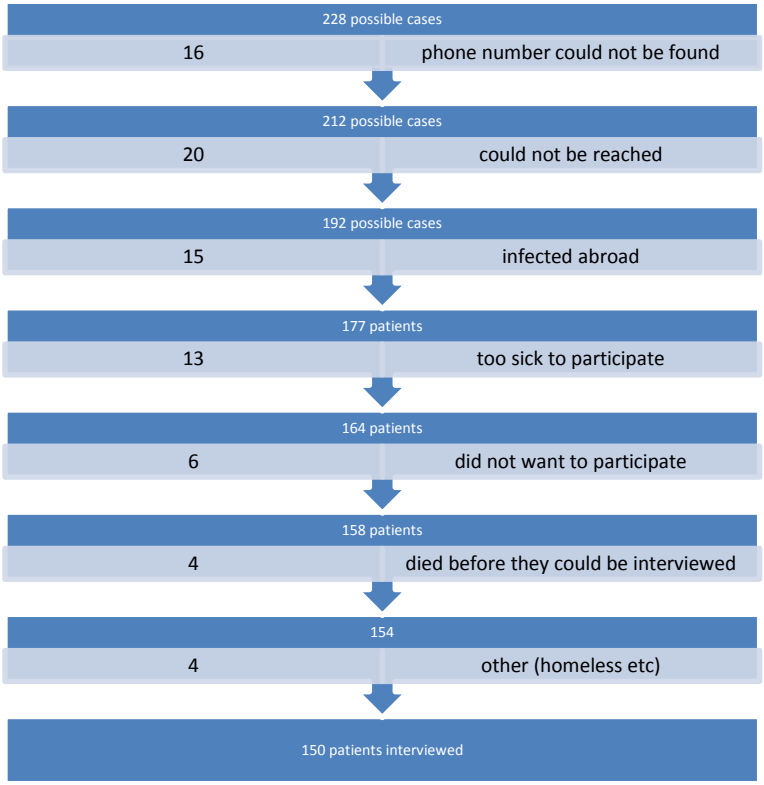


Figure 5.2: flow diagram for inclusion patients in the Telephone interview study

Analysis

The set up of this study is a prospective case-case study, this is a study set up in which a set of theory-based hypotheses, derived from literature, can be tested during a predetermined follow-up time.[262] The hypothesis tested in this prospective case-case study are described in Chapter 3, Table 3.1, in which traits that can possibly make certain phage types more successful than others are given. These hypothesis were then translated into a questionnaire, which can be found in the appendix of **Manuscript III**.

In **Manuscript III** descriptive analysis are given on the patients and their characteristics. Univariable and multivariable regression analyses were performed to describe the effect of antimicrobial use on outcome of resistance and underlying disease. Our outcome variable was resistance profile (pansuceptible, resistant, or multidrug-resistant), the independent variables included age category (<4 years, 4-17 years, 18-64 years, and 65 and older), gender, severe diarrhoea, (>7 days, yes/no), severe weight loss (>5kg lost, yes/no), hospitalisation due to infection (yes/no), medication received to treat infection (yes/no), vomiting more than 7 days (yes/no), feeling nauseated more than 7 days (yes/no), having stomach ache more than 7 days (yes/no), having fever more than 7 days (yes/no), having bloody faeces more than 7 days (yes/no), and having pain in joints for more than 7 days (yes/no).

Statistical tests of associations between resistance profile and disease outcome were computed by use of χ^2-test and Fisher's exact tests, as well as likelihood ratio tests in a logistic regression model, where appropriate.

Multivariate analysis was also performed; backward elimination as suggested by Hosmer and Lemeshow was used to determine the variables for the final regression model.[263] First, all univariable associations with a p-value of <0.25 were included in the multivariable model. Then, in a process of backward elimination, the least significant variables were exluded from the model one by one, until all variables in the model had a p-value of ≤0.05. During this process after each second variable was taken out of the model, each earlier eliminated variable was re-included in the model to assess whether the p-value of this variable would become significant after all.

Multicollinearity, confounding and interaction were all evaluated. In order to avoid multicollinearity, scores were created in which some variables were combined into one single variable. Variables excluded in the backward elimination procedure were re-entered in the final model to assess for confounding, by checking if the p-values or the estimates of the final model would change if the variable was reintroduced. Interaction terms of all biologically plausible variables (age, level of schooling, income, smoking, and self-reported stress), and all the variables left in the final model, were introduced in the final model to asses interaction.

All analyses were performed using the PROC GENMOD procedure used in SAS statistical software programme, version 9.3 (SAS Institute, Cary, NC).

6 Overview of Results

This chapter includes an overview of the main results obtained in this PhD study and a discussion of their significance. The chapter is divided into two sections: The first section discusses the long-term consequences of human antimicrobial use on the development of antimicrobial resistance as explored by the register-based studies, whereas the second section describes the short-term consequences of antimicrobial use on the health of the patient as investigated by the interview-based study.

6.1 Objective 1 - Assessment of the contribution of human antimicrobial use as a risk factor for acquiring an infection with an antimicrobial resistant *Salmonella* or *Campylobacter* strain

(**Manuscript I** and **Manuscript II**).

Salmonella

In the study period, between 1997-2005, a total of 22,602 *Salmonella* cases were reported, including 4,675 *Salmonella* Typhimurium, 12,152 *Salmonella* Enteritidis, and 5,776 other *Salmonella* serotypes. A total of 214,325 controls (1:10) were matched to these cases. Due to the low number of resistant isolates (e.g. no isolates of *S.* Enteritidis were resistant to third-generation cephalosporins, and no isolates belonging to the group 'other *Salmonella* serotypes' were resistant to amphenicols, see Table 6.1), the following groups of antimicrobials were excluded from the analysis: amphenicols, aminoglycosides, third generation cephalosporins, and the group named 'other antibacterials' with ATC-code J01X.

Table 6.1 Definition of the groups of antimicrobials, total number of susceptibility tested cases and prevalence of resistance to the classes of antimicrobials (1997 - 2005).

Groups of antimicrobials	*Salmonella* Typhimurium (%)	*Salmonella* Enteritidis (%)	other *Salmonella* serotypes (%)	Antimicrobials tested for in the susceptibility test
Total number of patients	4,675	12,151	5,776	
No. Susceptibility tested cases	4,534 (97.0)	4,195 (34.5)	1,679 (29.1)	
No. (of all cases) resistant to:				
aminoglycosides	1,292 (27.6)	37 (<0.1)	242 (4.2)	streptomycin, gentamicin, apramycin kanamycin, spectinomycin, neomycin
amphenicols	302 (6.5)	1 (<0.1)	0 (0)	Chloramphenicol, florfenicol
extended-spectrum penicillins	1,354 (29.0)	87 (<0.1)	236 (4.1)	ampicillin, amoxicillin-clavulanic acid
fluoroquinolones	164 (3.5)	326 (2.7)	460 (8.0)	ciprofloxacin, nalidixic acid
other antibacterials (ATC: J01X)	157 (3.4)	788 (6.5)	1 (<0.1)	collistin, nitrofurantoin, polymyxin, fosfomycin
sulphonamides and trimethoprim	1,713 (36.3)	57 (<0.1)	295 (5.1)	sulfamethoxazole, trimethoprim
tetracyclines	1643 (35.1)	69 (<0.1)	463 (8.0)	tetracycline
3rd generation cephalosporins	7 (<0.1)	0 (0)	4 (<0.1)	cefpodoxime, ceftiofur

Breakpoints for susceptibility testing are different for each year, see DANMAP 1997 - DANMAP 2005

In the year prior to *Salmonella* diagnosis, patients more frequently had a history of antimicrobial drug use than population controls. This was observed independent of serotype and susceptibility pattern, and was found for all classes of drugs but with some variation in estimates, see Table 6.2.

Table 6.2 Number of *Salmonella* cases and population controls exposed to antimicrobial drugs and the OR of *Salmonella* infection by history of antimicrobial drug use 2 weeks - 12 months prior to infection, Denmark 1997 - 2005; all *Salmonella* cases irrespective of susceptibility testing.

	No (%) exposed		OR of being exposed to antimicrobial drugs, OR 95%CI
	cases	controls	
Salmonella Typhiurium (n=4,675) exposure to:			
broad spectrum penicillins	601 (12.9)	3,998 (9.2)	1.56 (1.41 - 1.73)
fluoroquinolones	78 (1.7)	326 (0.8)	2.21 (1.70 - 2.86)
sulphonamides and trimethoprim	177 (3.8)	1,295 (3.0)	1.30 (1.10 - 1.54)
tetracyclines	64 (1.4)	440 (1.0)	1.32 (1.00 - 1.74)
Salmonella Enteritidis (n=12,151) exposure to:			
broad spectrum penicillins	1,202 (9.9)	9,169 (7.8)	1.33 (1.24 - 1.42)
fluoroquinolones	198 (1.6)	929 (0.8)	2.07 (1.24 - 1.42)
sulphonamides and trimethoprim	535 (4.4)	4,017 (3.4)	1.32 (1.20 - 1.45)
tetracyclines	194 (1.6)	1,402 (1.2)	1.33 (1.14 - 1.55)
other *Salmonella* serotypes (n=5,776) exposure to:			
broad spectrum penicillins	719 (12.4)	4,592 (8.5)	1.62 (1.47 - 1.77)
fluoroquinolones	186 (3.2)	387 (0.7)	4.55 (3.78 - 5.47)
sulphonamides and trimethoprim	317 (5.5)	1,815 (3.4)	1.72 (1.51 - 1.96)
tetracyclines	142 (2.5)	607 (1.1)	2.24 (1.85 - 2.71)

The ORs are adjusted for sex, age, county of residence, population density, income and schooling

Data pertaining to susceptibility tested strains only are given in Table 6.3. For several drugs (fluoroquinolones, tetracyclines, and broad-spectrum penicillins), there was an effect modification, i.e. resistant strains conferred a higher OR for salmonellosis than susceptible strains. The effect modification was statistically significant for fluoroquinolone-resistant *S.* Typhimurium and *S.* Enteritidis. The risk of being diagnosed with a fluoroquinolone-susceptible *S.* Typhimurium after having taken a course of fluoroquinolones was 2.0 times higher for patients than controls, whereas the risk of having a fluoroquinolone-resistant *S.* Typhimurium was 7.2 (2.0*3.6) times higher for patients than for controls. The logistic model is multiplicative and the product in the bracket is the main effect (OR 2.0 for susceptible strains) multiplied by the interaction term (OR 3.6 for the tested strain being resistant to the antimicrobial taken). Following the same argument for *S.* Enteritidis, the OR for acquiring a fluoroquinolone-resistant strain, after being exposed to a course of fluoroquinolones, was 1.7 for a susceptible strain whereas it was 4.5 (1.7*2.7) for acquiring a resistant strain (Table 6.3).

Table 6.3 Number of *Salmonella* cases and controls exposed to antimicrobial drugs and the OR of infection by history of antimicrobial drug use 2 weeks - 12 months before infection, analysed by susceptibility pattern of the infecting strain, Denmark, 1997 - 2005; only susceptibility tested cases included.

	No. (%) of exposed		Risk for salmonellosis after exposure (main effect), OR 95%CI	OR for resistant strain[a,b] (interaction term) OR 95%CI
	cases	controls		
Salmonella Typhimurium (n=4.628) exposure to:				
broad spectrum penicillins	595 (12.9)	3,967 (9.2)	1.50 (1.33 - 1.69)	1.17 (0.94 - 1.47)
fluoroquinolones	75 (1.6)	329 (0.8)	2.01 (1.53 - 2.64)	3.56 (1.22 - 10.34)
sulphonamides and trimethoprim	176 (3.8)	1,280 (3.0)	1.33 (1.07 - 1.65)	0.96 (0.67 - 1.36)
tetracyclines	62 (1.3)	435 (1.0)	1.03 (0.70 - 1.51)	1.72 (0.97 - 3.02)
Salmonella Enteritidis (n=4,412)) exposure to:				
broad spectrum penicillins	420 (9.5)	3,285 (7.9)	1.26 (1.12 - 1.41)	1.20 (0.54 - 2.69)
fluoroquinolones	68 (1.5)	347 (0.8)	1.69 (1.26 - 2.26)	2.65 (1.20 - 5.85)
sulphonamides and trimethoprim	184 (4.2)	1,401 (3.4)	1.26 (1.07 - 1.49)	2.64 (0.88 - 7.87)
tetracyclines	83 (1.9)	495 (1.2)	1.61 (1.26 - 2.05)	0.48 (0.06 - 3.80)
other *Salmonella* serotypes (n=1,619) exposure to:				
broad spectrum penicillins	183 (11.3)	1,224 (7.9)	1.42 (1.17 - 1.73)	1.53 (0.90 - 2.61)
fluoroquinolones	60 (3.7)	122 (0.8)	4.36 (2.97 - 6.41)	1.22 (0.56 - 2.66)
sulphonamides and trimethoprim	97 (6.0)	516 (3.3)	1.79 (1.38 - 2.32)	1.18 (0.60 - 2.32)
tetracyclines	38 (2.3)	154 (1.0)	2.30 (1.46 - 3.64)	1.03 (0.47 - 2.25)

The ORs are adjusted for sex, age, county of residence, population density, income and schooling

[a]: Cases that were exposed to antimicrobials 1 year before infection (and were infected with a strain that was resistant to the drug previously taken)

[b]: Statistically this was fitted as an interaction term, the total relative risk for infection with a drug-resistant *Salmonella* that is resistant to the drug previously taken can be estimated as the product of the two ORs

The OR for being diagnosed with *Salmonella* after exposure to one of the groups of antimicrobials in different time frames before infection was determined as well. The outcomes of this analysis for fluoroquinolones and broad-spectrum penicillins are given in Figure 1. This graph shows the OR, on a log-scale, plotted against the time since latest antimicrobial exposure before infection with *Salmonella*, in cubic splines. In general the highest excess risk was found in a time window up to 1 month before diagnosis, and levelled out and became relatively stable 24-6 months before diagnosis. Sulphonamides and trimethoprim were also associated with a time-dependent excess risk of being diagnosed, in particular for serotypes other than *S.* Enteritidis and *S.* Typhimurium. The ORs were 2.14 (95%CI: 1.43 – 3.23) for 0-2 weeks, 1.17 (95%CI: 0.74 – 1.87) for 2-4 weeks, 1.23 (95%CI: 1.01 – 1.50) for 1-6 months, and 1.40 (95%CI: 1.21 – 1.62) for 1-2 years before infection. The effect of age on outcome was also assessed, but no effect modification was found.

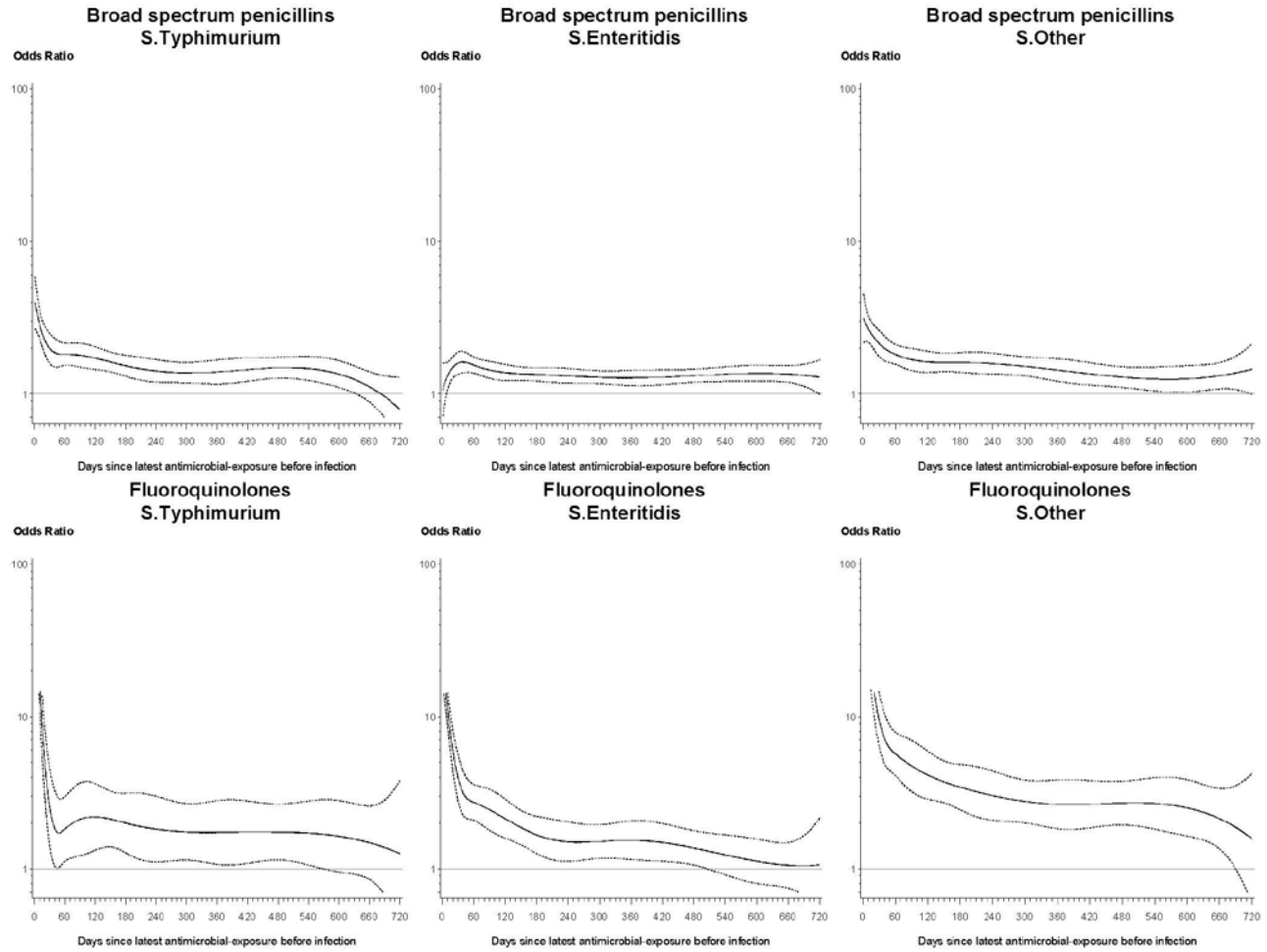


Figure 6.1: Cubic spline plots of the OR of being exposed to broad-spectrum penicillins and fluoroquinolones, 0-2 years before infection with *S.* Typhimurium, *S.* Enteritidis, or other *Salmonella* serotypes, 1997-2005, Denmark. The ORs are adjusted for sex, age, county of residence, population density, income and schooling.

Campylobacter

In the study period, between 1999-2005, a total of 31,699 *Campylobacter* cases were reported, no distinction was made between *C. coli* and *C. jejuni* cases. A total of 97,523 (1:10) controls were matched to these cases.

During the study, 10,275 (32.4%) of these cases were susceptibility tested against fluoroquinolones and macrolides. For these strains, we calculated excess odds of campylobacteriosis after drug exposure, as well as a multiplicative interaction term (the odds that the infective strain was resistant to the antimicrobial previously taken). A total of 459 strains were tested for a broader panel of

antimicrobial drugs as part of the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) surveillance (Table 6.4). However, due to the low number of strains examined, it was not possible to calculate the interaction terms for broad spectrum penicillins, sulphonamides and trimethoprim, and tetracyclines.

Overall, 21.7% of all cases were resistant to fluoroquinolones and 2.3% were resistant to macrolides, see Table 6.4. The prevalence of fluoroquinolone resistance was highest in adults 50-59 years of age and lowest in children 0-9 years.

Table 6.4 Age distribution and prevalence of resistance in *Campylobacter* strains from 10,475 cases examined for susceptibility for fluoroquinolones and macrolides, Denmark, 1999-2005

Age groups	Total no. of patients N (%)	Susceptibility tested[a] N (%)	Fluoroquinolone resistance[b] N (%)	Macrolide resistance[c] N (%)
0-9 years	4,076 (14.9)	1,556 (38.2)	224 (14.4)	25 (1.6)
10-19 years	3.039 (11.1)	1,150 (37.8)	231 (20.1)	21 (1.8)
20-29 years	6,841 (25.1)	2,750 (40.2)	613 (22.3)	57 (2.1)
30-39 years	4,971 (18.2)	1958 (39.4)	428 (21.9)	40 (2.0)
40-49 years	3,117 (11.4)	1,098 (35.2)	281 (25.6)	32 (2.9)
50-59 years	2,505 (9.2)	992 (39.6)	256 (25.8)	32 (3.2)
60-69 years	1,436 (5.2)	542 (37.7)	137 (25.3)	21 (3.9)
70-79 years	835 (3.1)	293 (35.1)	72 (24.6)	8 (2.7)
80 and older	456 (1.7)	136 (29.8)	26 (19.1)	2 (1.5)
Total	27,276 (100)	10,475 (38.4)	2,268 (21.7)	238 (2.3)

[a]: Overall $\chi 2$: 45.0, degrees of freedom: 8, P value: <0.001
[b]:Overall $\chi 2$: 69.8, degrees of freedom: 8, P value: <0.001
[c]: Overall $\chi 2$: 14.8, degrees of freedom: 8, P value: 0.06

Being diagnosed with campylobacteriosis was associated with an increased odds of exposure to a course of fluoroquinolones, macrolides, sulphonamides and trimethoprim, tetracyclines and broad spectrum penicillins up to 1 year before onset of disease (Table 6.5). This risk was highest for fluoroquinolones; OR 2.4 95%CI: 1.97 – 2.97. For fluoroquinolones, we found an effect modification, where being infected with resistant strains conferred a higher risk of diagnosis with *Campylobacter* than susceptible strains. The odds of being exposed to a course of fluoroquinolones was 2.4 times higher for cases diagnosed with a fluoroquinolone-sensitive *Campylobacter* than controls, whereas the odds of being exposed to a fluoroquinolone was 3.8 (2.4*1.6) times higher for cases with a fluoroquinolone-resistant *Campylobacter* infection than for controls. The logistic model is multiplicative, and the product between the brackets is the main effect (OR 2.4 for susceptible strains), multiplied by the interaction term (OR 1.6 for the tested strain being resistant to the antimicrobial taken). There was no interaction between macrolide exposure and being infected with a macrolide resistant *Campylobacter* (Table 6.5).

Table 6.5 Risk of *Campylobacter* diagnosis by exposure to a course of antimicrobial drugs 0-12 months before infection, Denmark, 1999-2005

Exposed to (up to 1 year before infection):	No (%) exposed		risk for campylobacteriosis after exposure (main effect) OR (95%CI)	OR for resistant strain[a,b] (inter-action term) OR (95%CI)
	cases	controls		
Broad spectrum penicillins	1,191 (11.6)	8.756 (9.0)	1.35 (1.25 - 1.45)	
Fluoroquinolones	183 (1.8)	658 (0.7)	2.42 (1.96 - 2.98)	1.61 (1.11 - 2.32)
Sulphonamides and trimethoprim	530 (5.1)	3535 (1.5)	1.48 (1.34 - 1.64)	
Tetracyclines	202 (2.0)	1,437 (1.5)	1.36 (1.16 - 1.59)	
Macrolides	1,158 (11.3)	7,642 (7.8)	1.48 (1.38 - 1.59)	1.04 (0.70 - 1.54)

The ORs were adjusted for sex, age, county of residence, level of schooling, and population density

[a]: Cases that were exposed to antimicrobials 1 year before infection (and were infected with a strain that was resistant to the drug previously taken).

[b]: Statistically, this was fitted as an interaction term, the total relative risk for infection with a drug-resistant *Campylobacter* that is resistant to the drug previously taken can be estimated as the product of the main effect and the interaction term

In the second part of the study, a time-dependent OR for previous exposure to antimicrobials was calculated in 6 different time-frames before onset of *Campylobacter* infection. The results of this analysis are given in Figure 1. This graph shows the OR plotted against the time since latest antimicrobial exposure before infection with *Campylobacter*, in cubic splines. The results for fluoroquinolones and macrolides were different, we found a positive association for previous fluoroquinolone use and the odds of being diagnosed with *Campylobacter*, whereas macrolides seem to have a protective effect.

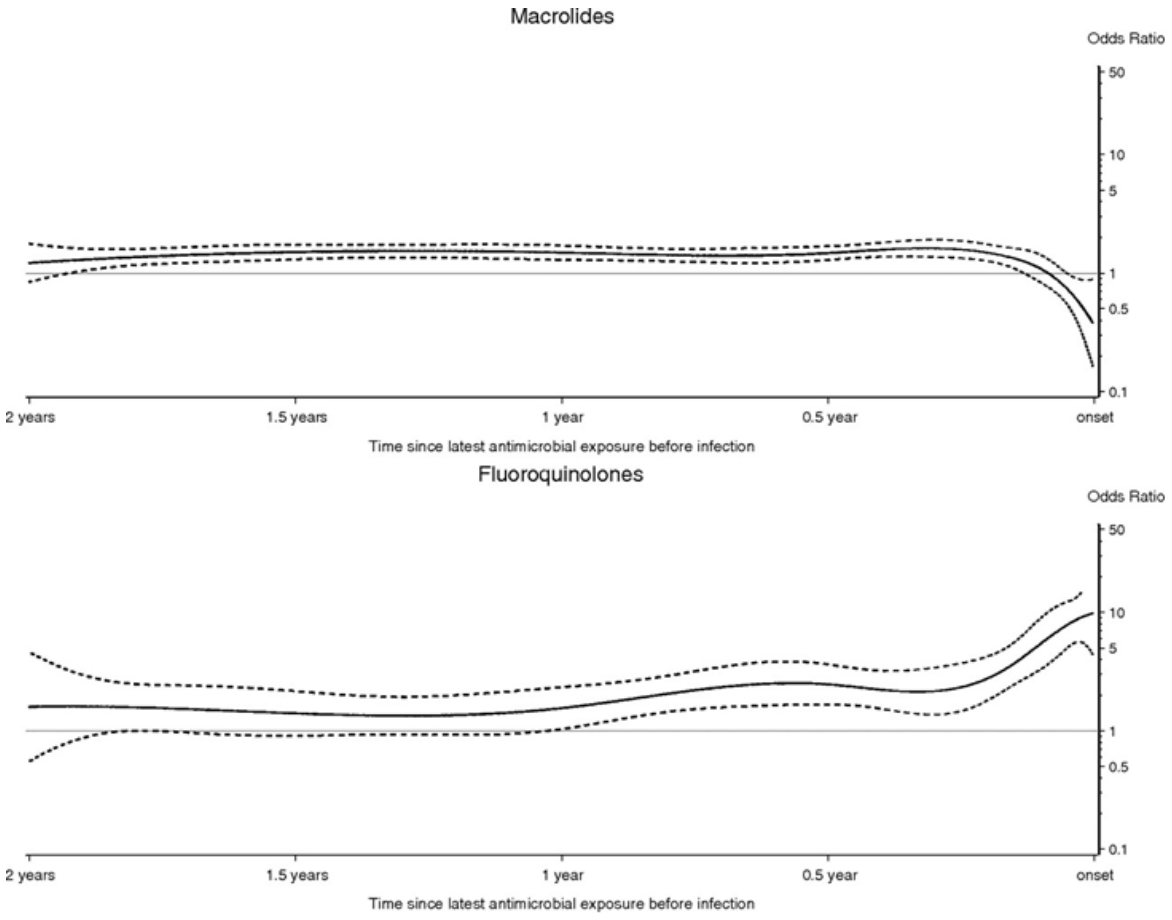


FIGURE 6.2 CUBIC spline plots of the odds ratio (OR) of being exposed to macrolides and fluoroquinolones 0–2 years before infection with Campylobacter, 1997–2005 Denmark. The ORs are adjusted for sex, age, county of residence, population density, income, and schooling.

Diagnosis with *Campylobacter* was associated with a decreased odds of macrolide consumption in the period close to diagnosis, while it was associated with an increased fluoroquinolone consumption in the same period, see Table 6.6. The effect of macrolides was more thoroughly examined to determine the duration of this protective effect and an analysis was performed for four different macrolides separately; erythromycin, roxithromycin, clarithromycin, and azithromycin. There was an excess odds of consumption of each of these drugs up to 1 year before infection, see Table 6.6. However, if the time between last exposure and *Campylobacter* infection is reduced to only the acute phase of infection (the estimated time before onset of disease and appearance in our database), exposure to macrolides became a protective factor (OR 0.72, up to 1 month before infection).

Table 6.6 Risk for *Campylobacter* diagnosis by timing of exposure to a course of antimicrobials 0-12 months before infection, Denmark, 1999-2005

Exposure to:	Up to 1 year before infection[a] OR (95%CI)	Up to 3 months before infection[b] OR (95%CI)	Up to 2 months before infection[b] OR (95%CI)	Up to 1 month before infection[b] OR 95%CI	Only the acute phase of infection OR (95%CI)
Erythromycin	1.40 (1.25 - 1.57)	Not enough statistical power to separate the analysis			0.06 (0.01 - 0.43)
Roxithromycin	1.63 (1.37 - 1.93)				0.39 (0.09 - 1.63)
Clarithromycin	1.62 (1.29 - 2.02)				0.83 (0.25 - 2.71)
Azithromycin	1.44 (1.30 - 1.60)				0.29 (0.11 - 0.79)
Macrolides grouped	1.48 (1.38 - 1.59)	1.16 (1.02 - 1.31)	0.98 (0.83 - 1.16)	0.72 (0.56 - 0.92)	0.30 (0.16 - 0.56)
Fluoroquinolones	2.42 (1.96 - 2.98)	2.54 (1.83 - 3.54)	2.65 (1.78 - 3.93)	2.94 (1.82 - 4.75)	10.32 (6.65 - 16.02)

The odds ratios (ORs) were adjusted for sex, age, county of residence, level of schooling income and population density

[a]: This period does not include the "acute phase" of infection

[b]: This period does include the "acute phase" of infection

6.2 Objective 2 – Comparison of clinical outcome of disease for *Salmonella* Typhimurium cases in relation to different susceptibility profiles (Manuscript III)

In the study period of January-June 2010, a total of 150 patients were enrolled in the study, and susceptibility testing was performed on all patient isolates. Isolates were tested for resistance against 19 different antimicrobials: ampicillin, apramycin, augmentin (amoxicillin-clavulanic acid), chloramphenicol, ciprofloxacin, cefotaxime, collistin, florfenicol, fosfomycin, gentamicin, nalidixic acid, neomycin, sulphonamide, spectinomycin, streptomycin, tetracycline, trimethoprim, ceftiofur, and mecillinam. The patients were grouped according to their resistance profile into three groups: pansusceptible (S), resistant (R), or multidrug-resistant (MR).

The median age of the cases was 36 years, with an interquartile range of 10-57 years. Twenty-six (17%) were under 4 years of age, and 24 (16%), were 65 or older. Common symptoms included: abdominal pain (>7 days, 81%), fever (>7 days, 73%), and severe diarrhoea (>7 days, 69%). We found no major differences in clinical outcome of disease between the 3 groups of patients (Table 6.7). Patients with a drug resistant *Salmonella* (R), had a higher odds for being hospitalised due to their *Salmonella* infection (OR 2.47 95%CI: 1.02 – 5.97), vomiting > 7 days (OR 2.61 95%CI: 1.10 – 6.19), and were more like to feel nauseous >7days (OR 2.87 95%CI: 1.26 – 6.54), than patients infected with a pansusceptible strain. We also found that there appeared to be more infants in the S than in the R (OR 3.09 95%CI: 1.08 – 8.82) or MR group (OR 4.24 95%CI: 1.41 – 12.77).

Table 6.7 Odds for severity of disease per resistance profile (three levels), 150 *S.* Typhimurium patients, January-June 2010, Denmark

	Three resistance profiles						All patients	
	Pansusceptible (S)*		Resistant (R)*		Multidrug-resistant (MR)*			
	N	OR (95%CI)	N	OR (95%CI)	N	OR (95%CI)	N	(%)
Total number of patients	49		48		53		150	100
Median age (interquartile range)	26	(3-51)	49	(23-65)	29	(11-55)	36	(10-57)
Age category[a]		1 (reference)		3.04 (1.40-6.56)		2.23 (1.06-1.68		
Infants (<4y)	15	3.44 (1.36-8.70)	6	0.52 (0.19-1.46)	5	0.44 (0.15-1.30)	26	17.3
Children (4-17y)	8	1.35 (0.50-3.64)	4	0.37 (0.11-1.19)	11	1.69 (0.66-4.36)	23	15.3
Adults (18-64y)	21	1 (reference)	27	1 (reference)	26	1 (reference)	74	49.3
Seniors (=> 65y)	5	0.66 (0.22-2.01)	10	1.24 (0.49-3.18)	9	1.11 (0.43-2.88)	24	16.0
Gender (female)	25	1 (reference)	23	1.13 (0.51-2.51)	26	1.08 (0.50-2.53)	74	49.3
Any underlying comorbidity	12	1 (reference)	21	2.40 (1.00-5.70)	27	3.20 (1.38-7.45)	60	40.0
Median days of diarrhoea (interquartile range)	14	(7-15)	10	(7-14)	14	(8-20)	13	(7-15)
severe diarrhoea (>7 days)	33	1 (reference)	27	0.62 (0.27 -1.43)	43	2.09 (0.84 - 5.62)	103	68.7
Weight loss (>5kg)	8	1 (reference)	14	2.05 (0.76-5.58)	13	1.73 (0.63-4.71)	35	23.3
hospitalised due to infection	11	1 (reference)	20	2.47 (1.02- 5.97)	18	1.68 (0.70 - 4.04)	49	32.7
Received medication to treat infection	22	1 (reference)	29	1.87 (0.84-4.20)	25	1.10 (0.50-2.39)	76	50.7
History of antimicrobial use[b]	20	1 (reference)	23	1.33 (0.60-2.98)	25	1.30 (0.59-2.83)	68	35.3
Vomiting (>7 days)	12	1 (reference)	22	2.61 (1.10-6.19)	20	1.87 (0.79-4.40)	54	36.0
Nausea >7 days)	18	1 (reference)	30	2.87 (1.26-6.54)	27	1.79 (0.81-3.95)	75	50.0
Abdominal pain (>7 days)	39	1 (reference)	41	1.50 (0.52-4.33)	42	0.98 (0.38-2.56)	122	81.3
Fever (>7 days)	36	1 (reference)	35	0.97 (0.40-2.39)	39	1.01 (0.42-2.43)	110	73.3
Bloody faeces (>7 days)	19	1 (reference)	20	1.1 (0.50-2.54)	20	0.96 (0.43-2.13)	59	39.3
Pain in joints	13	1 (reference)	11	0.82 (0.33-2.08)	15	1.09 (0.46-2.61)	39	26.0

* S: pansusceptible *Salmonella,* R: *Salmonella* resistant to 1-3 antimicrobials, MR: *Salmonella* strains resistant to =>4 antimicrobials

[a]: Age category was analysed separately within the different susceptibility profiles with adults as the reference value

[b]: Antimicrobials were not prescribed for treatment of current infection, history up to 6 months.

In Table 6.7, it can be seen that age category is associated with resistance profile. Since age is a known confounder in epidemiological research, and not a biological explanation, we assessed the link between comorbidity, age category and resistance profile a bit further. We found both age and resistance profile to be explanatory variables for comorbidity (as defined in chapter 5, see Table 6.8), explaining the effect seen in Table 6.7.

Table 6.8 Relation between comorbidity and resistance profile, crude outcomes and adjusted for age category

Resistance profile	Comorbidity present	N (%)	Crude OR (95%CI)	adjusted OR* (95%CI)
S	Yes	12 (24)	1 (Reference)	1 (Reference)
	No	37 (76)		
R	Yes	21 (44)	2.40 (1.01 - 5.70)	1.73 (0.68 - 4.35)
	No	27 (56)		
MR	Yes	27 (51)	3.20 (1.38 - 7.45)	2.62 (1.07 - 6.37)
	No	26 (49)		
< 4 years	Yes	4 (15)	1 (Reference)	1 (Reference)
	No	22 (84)		
4-17 years	Yes	6 (26)	1.94 (0.47 - 7.99)	1.52 (0.36 - 6.48)
	No	17 (74)		
18-64 years	Yes	33 (43)	4.12 (1.30 - 13.12)	3.37 (1.03 - 11.01)
	No	44 (57)		
=> 65 years	Yes	7 (29)	13.36 (3.35 - 53.18)	10.89 (2.66 - 44.61)
	No	17 (71)		
Total				

*Comorbidity by resistance profile and for age category

**: Comorbidity is a yes/no variable consisting of having any of the following underlying diseases: asthma/bronchitis, heart and circulation disease, intestinal illness, recurrent diarrhoea, liver disease, diabetes, connective tissue disease, kidney problems, cancer, chronic infection, other disease.

6.3 Objective 3 – Examination of the association between clinical outcome of infection and previous antimicrobial use (Manuscript III)

This study used the same patient material as described above. As described in chapter 5, data on clinical outcomes were obtained through telephone-conducted interviews and logistic regression was applied to assess the association between clinical outcome of disease and previous antimicrobial use. The age- and gender distribution, as well as clinical outcomes of disease, are presented in Table 6.7.

In total, 68 patients (45%) stated that they had a history of antimicrobial treatment in the six month period preceding infection. Patients with a history of treatment tended to have a higher odds of infection with a resistant or multidrug-resistant *Salmonella* strain, but these differences were not significant (Table 6.7). Furthermore, a simple analysis, combining resistant with multiresistant strains into one group, did also not confer statistical significance (OR 1.31 95%CI: 0.62 – 2.79).

The relation between history of previous antimicrobial use (up to 6 months before infection) was examined in a multivariable analysis with the different clinical outcomes of disease. The results of this analysis are given in Table 6.9. Patients who had been exposed to a course of antimicrobials, not related to the *Salmonella* infection, up to half a year before they got infected with *Salmonella*, had a higher odds of being hospitalised (OR 2.00, 95%CI 0.97 – 4.11), lose more than 5kg of weight (OR 2.01, 95%CI 0.84 – 4.82), and receive treatment for the current event of salmonellosis (OR 7.56, 95%CI 3.42 – 16.70). The other outcomes of clinical illness (>7days of diarrhoea, vomiting, feeling

161 nauseated, abdominal pain, fever, bloody faeces and pain in joints) were not associated with
162 previous unrelated use of antimicrobials.

Table 6.9 Crude and adjusted odds ratios for people who received antimicrobials in the past 6 months prior to *Salmonella* infection

		History of antimicrobial use, unrelated to the current salmonellosis		Odds Ratio for severe outcome (95%CI)	
Consequence of infection:		Yes	No	Crude	Adjusted*
Prescribed antimicrobials	yes	52	24	7.85 (3.77 - 16.78)	7.56 (3.42 - 16.70)
	no	16	58	1 (reference)	1 (reference)
Hospitalised	yes	28	21	2.03 (1.02 - 4.06)	2.00 (0.97 - 4.11)
	no	40	61	1 (reference)	1 (reference)
Weight loss (>5kg)	yes	22	39	2.43 (1.09 - 5.48)	2.01 (0.84 - 4.82)
	no	56	13	1 (reference)	1 (reference)

* We adjusted for age category, income, level of schooling, self-reported stress, and smoking

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7 Discussion and Conclusions

In this chapter, the pros and cons of the study designs used, and the results of the research objectives will be discussed. In the end of this chapter the general conclusions are given and some future perspectives will be outlined.

7.1 Strengths and limitations of database research

Since the CPR-registry came in place in 1968, many more registries have been established in Denmark, currently a total of 59 clinical databases, of which 51 are national registries, have been adopted by the National Board of Health.[264] All these databases use people's CPR-number as an individual identifier for the data.

The strength in using large national registries, like the NREP or the Danish Prescription Database, is the data amount and the time-span of the data-collection. The Danish registries are well kept and often complete, and by combining these data with microbiological susceptibility data or isolate characterisations, it provides numerous opportunities for large cohort or case-control studies. The unique identification number (CPR-number) enables linkage with other registries and allows follow-up on individuals on long-term disease consequences.

Using these databases also has another advantage; the data is already collected, which saves time, money, and effort for conducting studies. As mentioned before, these national registries are large, and often complete which enables very large sample-sizes, enabling high statistical power to estimate precise outcome measures of rare diseases and occurrences. Another advantage is that selection bias does not play a major role for enrolling in the study. All data entered in these databases are included on the same grounds, albeit a small part of the Danish population has refrained from being documented in these databases ("forskerundtagelse").

However, performing registry-based studies also has some disadvantages. Many registries are established for administrative purposes, and data validity can be hard to assess, which can be a source of information bias. In addition, the type of data to be collected is predetermined, with the possible consequence that discrete outcomes and possibly important confounders cannot be assessed. For example, many procedures and supplementary diagnosis may not be available from the NREP.

7.2 Objective 1

In **objective 1** the contribution of human antimicrobial use as a risk for acquiring an infection with *Salmonella* or *Campylobacter* was assessed. The risk for acquiring an infection with either bacteria after previous use of antimicrobials was quantified, additionally, the risk for acquiring an infection resistant to the antimicrobial previous taken, was calculated.

As discussed in chapter 3, a person is more vulnerable to infection with a pathogen after exposure to a course of antimicrobial drugs, because the colonisation resistance is temporarily lowered due to the effect of the antimicrobial on the gut flora in the intestines i.e. the competitive effect. When the

person in addition is exposed to an antimicrobial resistant pathogen, which is resistant to the antimicrobial taken, the selective effect applies. The patient has an increased risk of infection with this antimicrobial resistant pathogen, which is an additive to the competitive effect. This also applies if a person is an asymptomatic carrier of such an antimicrobial resistant pathogen.[265, 266] In **manuscript I** and **manuscript II**, we were able to quantify these effects. For *Salmonella* we visualised the selective effect in Figure 6.1, and for *Campylobacter* in Figure 6.2. The premise of our model is that the selective effect is a multiplication of the ORs given in Table 6.3 and Table 6.5 respectively: not only should the patient be exposed to an antimicrobial, but the infective strain also has to be resistant to the antimicrobial exposed to.

For calculation of the overall or competitive effect (i.e. being exposed to a course of any antimicrobial before infection), all *Salmonella* and *Campylobacter* cases were taken into account, independent on whether they were susceptibility tested or not. The results indicate that a history of antimicrobial drugs confers an increased risk of being diagnosed with either *Salmonella* or *Campylobacter*, where the risk appears to be present up to at least 1 year post-treatment, possibly even longer. The effect may depend on several factors, including the spectrum of the antimicrobial, the antibacterial effect of its metabolites and its pharmacokinetics. Broad-spectrum penicillins, sulphonamides and trimethoprim are excreted mainly through the urine, and therefore have a lesser impact on the gut flora.[267, 268] This is also the case for most fluoroquinolones, but nevertheless, a significant part of this group of antimicrobials is excreted in the bile. Also, some of the active metabolites of fluoroquinolones are excreted through the biliary route.[269] This implies that fluoroquinolones and their metabolites have an impact on the gut flora by passing through the gastrointestinal tract, which explains why fluoroquinolones are the first drug of choice to treat gastrointestinal infections, and why the most consistent results were obtained for this drug. Tetracyclines are excreted via both the biliary and the urinary pathways. This drug also reaches fairly high concentrations in the gastrointestinal tract in its unchanged form.[270] We found that diagnosis with *Campylobacter* was associated with reduced odds of recent history of macrolides, compared with controls. The pharmacokinetics of macrolides are well described in the literature. The half-life of macrolides is different for each of the drugs included in the study with erythromycin having the shortest half-life (<4 hours), roxithromycin and clarithromycin an intermediate half-life (4-24 hours) and azithromycin having the longest half-life (>72 hours).[271] However, the protective effect of the drugs last at least 4 weeks, see Figure 6.2. The protective effect of macrolides, as a group, in the acute phase and 0-2 weeks before the index date (OR 0.72, 95%CI 0.56 – 0.92) was strong. The acute phase was specifically constructed so we could not confuse drugs prescribed as early empirical treatment as a risk factor for acquiring *Campylobacter*. It is interesting that a protective effect is seen in all the macrolides (Table 6.6), even though the kinetics of each of the macrolides is quite different. One explanation for this phenomenon is that macrolides, especially azithromycin, become trapped in the intracellular lysosomes of phagocytic cells. This intracellular uptake is easily reversible for erythromycin and clarithromycin but is extremely slow and probably not completely reversible for azithromycin.[272, 273] It is even possible that part of the azithromycin metabolites remain trapped in the lysosome until the cell dies and the drug is finally released. Whether this is an explanation for the long-term protective effect remains unknown and should be further investigated. Another possibility could be that a treatment with macrolides deplete the gut from nutrients that are

essential for the growth of *Campylobacter* spp.. It is obvious that it is beyond the scope of an epidemiological study to address such biological hypothesis in details.

The fact that macrolides are mostly prescribed in the winter season for respiratory infections, and *Campylobacter* infections are most common in the late summer could create a seasonal confounding effect. Still, we adjusted for season by matching on the index date of the case and we therefore do not find this to be a likely explanation of the association observed. Furthermore, several other antimicrobial drugs are prescribed more commonly in the winter season as well, without causing a similar pattern.

The effect seen in the first half year before infection, of Figure 6.1 and Figure 6.2, is a combination of the competitive effect and the selective effect. An effect of treatment (protopathic bias) can be seen if a patient had an antimicrobial prescription for diarrhoeal symptoms before the actual stool sample was taken. However, we accounted for this by introducing an index date, which excluded the 'acute phase' of exposure and added an extra week to allow for the sample to show up in the database, see Figure 5.1.

For *Salmonella*, Figure 6.1, the OR for being diagnosed after a treatment with antimicrobials, up to half a year before infection, is higher for patients than for controls in all groups except for exposure to broad-spectrum penicillins in *Salmonella* Enteritidis. The effect seen might be explained by the low percentage of resistance to this group of antimicrobials in *S*. Enteritidis. Resistance levels to broad-spectrum penicillins are 3-6% in *S.* Enteritidis and 44-56% in *S.* Typhimurium.

In both *Salmonella* and *Campylobacter*, the effect of taking a course of antimicrobials in the period 6 months – 2 years before infection remains high, but stabilises. This effect can probably be ascribed to selection bias (i.e. the effect that some people are more likely to be tested than other people, possibly due to underlying comorbidity). A history of antimicrobial drug use may also be an indicator of increased use of healthcare and thus a marker of frailty and therefore might contribute to this effect. Furthermore, a *Salmonella* or *Campylobacter* may develop diarrhoea due to the non-specific effect of the antibiotic and may get sampled on this basis. However, it cannot be ruled out that some antimicrobial drugs may have a long-term effect on the gut flora and thus render patients more susceptible to bacterial infections with either *Salmonella* or *Campylobacter*.

In **Manuscript II**, we also investigated the effect of age on prevalence of resistance. Some statistically significant differences in the number of samples susceptibility-tested in each age-group; The prevalence of fluoroquinolone resistance was highest in adults 50-59 years of age, and lowest in children. This is effect is likely to be explained by two factors: 1) it is not common to prescribe fluoroquinolones to children, and 2) the differences in travel behaviour between the age groups. In 2008, 27.4 % of *Campylobacter* cases were travel related cases[274], and were associated with a higher prevalence of antimicrobial resistance.[161] However, matching on age should have accounted for this problem.

This study designs used in **Manuscript I** and **Manuscript II**, is subject to a number of limitations. The first one is already described above as selection bias, due to this being registry based study, and patients are only enrolled if they have handed in a stool sample, and these are likely to be the more severe cases. Another limitation is that some of the antimicrobials are mainly used in hospital setting,

and these data are not included in the Danish prescription registry. We can only speculate that the impact of antimicrobial drugs on the occurrence of antimicrobial resistance would have been higher, if we could also have included these data. Still in Denmark, 90% of the prescribed daily defined doses are administered in the primary health care sector.[161] We did not adjust for co-morbidity in this study, but in a similar study, performed by Gradel et. al.,[275] this was taken into account and it did not affect the outcomes markedly. Therefore it is not likely that adjusting for co-morbidity in this study would have had any major impact on our results.

7.3 Prospective case-case interview study

The major strength of using this study design is that one can include all the relevant variables and confounders.

A case-case study design has several advantages over a regular case-control study. Firstly by comparing the phage types of interest with other *S*. Typhimurium cases, only risk factors that are specifically associated with the phage type under investigation are identified, whereas general risk factors for acquiring an infection with *S*. Typhimurium are filtered out. Secondly, the risk of selection-bias is reduced as both case-cases and case-controls would be enrolled through the same surveillance system. Finally, the willingness to participate would be the same in either group, whereas it is usually difficult to get healthy controls from the general population to participate.

The limitation of the study design in comparison to a registry-based study is that it is costly and time-consuming, and, it might also be unpredictable. As was shown in the methods chapter, we needed 50 case-patients of each phage type and 100 case-controls, and we only had half a year planned to do all the interviews. At the time we realised that we could not obtain the required amount of cases, we did not have the possibility to extend the interview-time. This resulted in a modification of the study set-up.

7.4 Objective 2

In contrast to other studies, we were not able to show convincingly that infections with antimicrobial resistant *Salmonella* have a worse outcome of disease than infections with pansusceptible *Salmonella* strains (S). Though we did find that patients with a resistant (R) susceptibility profile had higher odds of being hospitalised due to their salmonellosis, experience abdominal pain and feeling nauseated than patients with a pansusceptible *Salmonella*. We found no increasing trend with increasing antimicrobial resistance (S versus multiresistant; MR). It is possible that even the higher risk of vomiting and nausea are a spurious observation. But it is also possible that the observation is due to the mix of phage types in the groups. It is known that some phage types are better suited to cause disease in humans than others, it might also be possible that some of these phage types are more likely to cause disease in humans as well. Further studies could clarify this in the future. Another explanation for these findings could be that patients with a worse outcome of disease did not receive correct empirical treatment. This hypothesis is hardly tested in literature, but it is unlikely to play a large role in our study: first drug of choice for empirical treatment of diarrhoeal disease is ciprofloxacin (a fluoroquinolone), and fluoroquinolone resistance only occurred twice in our study

population. In patients with a travel-history, fluoroquinolone resistance is more common[161], but these were excluded from our study.

Other investigators have shown that being infected with an antimicrobial resistant *Salmonella* leads to a worse outcome of disease, including higher risk of bacteraemia[276], higher mortality[277, 278], excess hospitalisation[279, 280], and more infections due to therapy failure.[281] Most likely this difference is due to our study set-up which focused on softer outcomes than the abovementioned ones. The studies of Helms (2003)[282], Lee (1994)[283], Giamarello-Bourboulis (2006)[284], and Varma (2005)[285] et. al., included hospitalised patients, or used hospitalisations as an indicator for severe disease, whereas we included disease outcomes such as days of diarrhoea, vomiting, weight loss, and other events not necessarily requiring hospitalisation. Furthermore, we included a lower number of patients compared with the higher-powered registry based. Finally, interviews were conducted relatively quickly after onset of disease, and some of the patients were still ill during the interview, and we did not follow up to look at more long-term outcome of disease including the possibility for a more severe disease development.

It is possible that we missed out on the most severely ill people; however, the interviewers called patients in the evening hours (4-9 pm), thus reducing the risk of missing people due to their working-hours. The interviewers managed to interview 66% (or 70% after omitting 15 patients who were infected abroad and thus not eligible to interview). This is a satisfactory participation rate compared to other similar studies[286, 287], but nonetheless, it is likely that very ill people would not, or could not, participate. The interviewers did make short notifications of the reasons that patients did not participate, and from this list we derived that 13 patients were too ill to participate when they were called.

We also found that older patients seemed more likely to suffer from underlying comorbidity, and to be diagnosed with a resistant or multidrug-resistant *Salmonella* infection (Table 6.8). Increasing age is a classic confounder for underlying comorbidity in epidemiologic studies. Older people tend to accumulate an increasing number of health complaints and are therefore more prone to visit a GP or a hospital, and are also more likely to be tested for infections and to be subsequently treated with antimicrobials. As is shown in **Manuscript I** and **Manuscript II**, people treated with antimicrobials appear to be more likely to be diagnosed with *Salmonella* or *Campylobacter*.

7.5 Objective 3

A history of antimicrobial treatment, unrelated to the current salmonellosis, up to six months before infection was associated with a more severe clinical outcome of disease. Patients, who had received a course of antimicrobial treatment in the past, had higher odds for severe weight loss (>5 kgs), hospitalisation and antimicrobial treatment for their current salmonellosis. There are several possible explanations for this observation. It is possible that this is an extension of the competitive and selective effect of antimicrobial treatment (**Manuscript I** and **Manuscript II)**, where past antimicrobial treatment depletes or changes the composition of the gut flora in a way that increases severity of infection. Alternatively, a past history of treatment could be an indicator or proxy of a vulnerable patient**.**

We found that one of every two patients (51%) was treated with antimicrobial drugs. This was a surprise, since Denmark is known for a prudent use of antibiotics. Elderly patients, over 60 years, used more antimicrobials than younger patients in our study, and this coincided with a higher percentage of these patients being admitted to a hospital for their infection. It is likely to be due to the fact that older people have a reduced functioning of the immune-system and that elderly get dehydrated more easy than younger people[288], and are therefore more likely to be seriously affected by an infection like *Salmonella*.[289] However, of the youngest age-group (<4 years), 35% (9 out of 26) were treated with antibiotics, which is a cause for concern. Antimicrobial treatment for salmonellosis is not recommended, unless absolutely necessary. On the other hand, more mild cases presenting with mild diarrhoea and other mild symptoms are unlikely to be (stool-) sampled, and are therefore not represented in our study.

Another explanation for our findings could be that asthma/bronchitis were the most common underlying disease in our study. These patients are more likely to suffer from upper-respiratory illnesses[290], which might be treated with antimicrobials adding to the antimicrobial use of our study-population. Unfortunately, the clinical indication for the earlier prescribed antimicrobial course was not investigated.

7.6 General Conclusions and future perspectives

In this thesis, multiple hypotheses were tested. The first conclusion is that with an increasing number of antimicrobial prescriptions, the number of clinical infection with antimicrobial-resistant *Salmonella* and *Campylobacter* are likely to rise as well. The use of broad-spectrum penicillins, sulphonamides, trimethoprim and tetracyclines, and in particular fluoroquinolones up to half a year before infection, was associated with a higher risk of infection with both *Salmonella* and *Campylobacter*. In addition, patients who were exposed to fluoroquinolones had a significantly higher risk for acquiring a *Salmonella* or *Campylobacter* infection that was resistant to the drug previously taken. In *Salmonella,* this tendency was also found for broad-spectrum penicillins, sulphonamides and trimethoprim, and tetracyclines.

Registry-based studies are very efficient to assess hypotheses like these. The enormous amount of data provides good statistical strength to perform such analysis. We did not have enough statistical power to calculate this effect in some of the classes of antimicrobials that were initially included in the study. By now, eight more years of data have been included in the registries, and perhaps this could be re-examined.

The second conclusion is that we were not able to demonstrate major negative outcomes of *S*. Typhimurium infection when comparing patients infected with strains classified in three levels of antimicrobial drug resistance. Nevertheless, this effect was found in many other large-scaled studies. Our negative finding is likely to be a limitation of the design of the study rather than a true absence of a detrimental effect. Therefore, it is recommended to perform a high-powered study taking advantage of electronic health records or other databases. As these databases are more appropriate to address research questions regarding the public health effect of antimicrobial drug resistance than work-intensive interview studies, such as the one presented in this thesis.

We also found that people who had received a course of antimicrobials, unrelated to their current salmonellosis, up to six months before they got ill, had higher odds for hospitalisation, severe weight loss (>5kgs lost), and treatment with antimicrobials for their current infection. To our surprise, we found that half of the patients interviewed, had received an antimicrobial treatment for the *Salmonella* infection. This is a concern in light of the need to advocate prudent use of antimicrobials. As mentioned in the discussion of the results, mild cases of salmonellosis (or any bacterial gastroenteritis for that matter) are not sampled, and are therefore not represented in the national registry of enteric pathogens, and thus not included in our study. To assess the actual number of prescriptions prescribed for diarrhoeal diseases, GPs could be approached.

The overall, and most important conclusion of this thesis is that human antimicrobial use interacts in many ways with the risk of being infected with antimicrobial drug resistant strains of *Salmonella* and *Campylobacter, and that it may be associated with severity of infection as well*. The protective role of macrolides with regards to *Campylobacter* infection adds another layer of complexity to these interactions. Prudent use of antimicrobial drugs should always be advocated in human health practices. And future studies should point out whether the associations found in this thesis are also present in other pathogens.

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Manuscript I:

The interaction between prior antimicrobial drug exposure and resistance in human *Salmonella* infections

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J Antimicrob Chemother 2010; **65**: 1819–1825
doi:10.1093/jac/dkq176 Advance Access publication 27 May 2010

Journal of Antimicrobial Chemotherapy

The interaction between prior antimicrobial drug exposure and resistance in human *Salmonella* infections

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Received 24 September 2009; returned 2 December 2009; revised 27 January 2010; accepted 27 April 2010

Objectives: The use of antimicrobial drugs for food animals selects for resistant non-typhoid *Salmonella* strains, but human consumption of antimicrobial drugs may also increase the risk of subsequent infection. The aim of this study was to determine the risk of salmonellosis attributable to human consumption of antimicrobial drugs in a case–control study of 22 602 laboratory-confirmed *Salmonella* infections, diagnosed in Denmark between 1997 and 2005.

Methods: A population registry-based case–control study, using several Danish databases: the National Prescription Database; the National Registry for Enteric Pathogens; the Civil Registry System; and the Integrated Database on Labour Market Research.

Results: Exposure to trimethoprim, sulphonamides, broad-spectrum penicillins, tetracyclines and fluoroquinolones, during the year prior to diagnosis, was associated with an increased risk of non-typhoid *Salmonella* infection. Overall, the highest risk was associated with the prior use of fluoroquinolones. This risk increased as the time window of exposure approached the infection date. Previous use of fluoroquinolones was associated with an odds ratio (OR) of 4.55 [95% confidence interval (CI): 3.78–5.47] for *Salmonella* serotypes other than *Salmonella* Typhimurium or *Salmonella* Enteritidis, an OR of 2.21 (95% CI: 1.70–2.86) for *Salmonella* Typhimurium and an OR of 2.07 (95% CI: 1.76–2.42) for *Salmonella* Enteritidis. In particular for fluoroquinolones, there was an interaction between the pathogen resistance pattern and a history of antibiotic drug use.

Conclusions: The increasing use of antibiotics, particularly fluoroquinolones, is likely to result in increased incidence of foodborne infections with drug-resistant *Salmonella*.

Keywords: antimicrobial consumption, antimicrobial drug resistance, registry-based study, competitive effect, selective effect, case–control study

Introduction

In many countries, antimicrobial drug resistance in foodborne pathogens is recognized as an increasing problem.[1–4] The widespread use of antimicrobial drugs for food animals is the major factor for the selection and dissemination of resistance in zoonotic foodborne pathogens such as non-typhoid *Salmonella enterica* serotypes (hereafter named *Salmonella*).[5–9] Zoonotic bacteria are usually already resistant at the time of human exposure from food sources. However, it has also been suggested that human antimicrobial drug use is an additional risk factor for antimicrobial drug resistance in foodborne pathogens. Thus, several epidemiological studies show that pre-infection antimicrobial therapy (for indications other than gastroenteritis) is a risk factor for infection with antimicrobial drug-resistant bacteria of animal origin. The use of antimicrobial drugs may cause a transient increase in susceptibility to infection after exposure to a foodborne pathogen. This susceptibility may be separated into a so-called 'competitive effect' and a 'selective effect'; the selective effect offers a specific advantage for a resistant pathogen.[10,11] In other words, drug-resistant gastrointestinal pathogens preferentially cause illness in persons receiving antimicrobial drugs for any medical condition. This issue has been studied in outbreak investigations and in case–control studies of sporadic infections, but little research has been carried out to investigate the long-term consequences of human antimicrobial drug use for acquiring resistant foodborne pathogens. Recently, Gradel *et al.*,[8] found that *Salmonella* patients had an excess risk of being exposed to antimicrobials in the year before infection compared with their controls. Whereas this

observation could partly be ascribed to a long-term competitive effect of broad-spectrum antimicrobial use, the study also indicated that higher antimicrobial use might be a marker for frailty and therefore an indicator of patients who are more susceptible to becoming infected with, or are more likely to be diagnosed with, *Salmonella*. The study did not have sufficient statistical power to address whether a history of consumption of antimicrobial drugs conferred an excess risk of getting infected with drug-resistant *Salmonella*.

In the present nationwide registry-based study, we analysed the association between pre-infection exposure to antimicrobial drugs and laboratory-confirmed *Salmonella* infection. Due to the size of the study, we were able to address the role of drug resistance, and determine the long-term consequences of a history of antimicrobial use by different groups of antimicrobials. We were, on this basis, able to consider three different selective effects for acquiring resistance: the selective effect; the competitive effect; and the probable effect of selection bias or frailty.

Materials and methods

Registers

During the study period, from 1997 to 2005, all cases of *Salmonella* infection confirmed by faecal culture or by a culture from a normally sterile site were reported to Statens Serum Institut and data entered into the National Registry for Enteric Pathogens.[12,13] Susceptibility testing was performed on a sample of submitted strains by the Unit of Gastrointestinal Infections of Statens Serum Institut.[14] For surveillance purposes, all isolates of serotype Typhimurium were targeted for susceptibility testing whereas we examined a random sample of other serotypes (with Enteritidis as the most common). In total, 46% (10408 samples) of the strains included in the present study were susceptibility tested; ranging from 97% of the *Salmonella* Typhimurium strains to 29% of other serotypes (Table 1). The data were entered into these databases including a unique personal identification number used in the Danish Civil Registry System. The personal identification number enables linkage between the different surveillance and public health databases.[15] Information on county and municipality can be directly deduced from the Civil Registry System.

To obtain data on exposure to antimicrobial drugs prior to the *Salmonella* infection, we used the National Prescription Database. This database contains information on all prescriptions provided by general practitioners, and is collected at local pharmacies. Data were entered using the Civil Registry System number and included the generic name of the drug, the ATC code, dosage, price and date of issue.

To control for the confounding effect of education and socio-economic status, we obtained data on schooling and income from the Integrated Database on Labour Market Research.[16]

Case patients and controls

Patients enrolled in our study were all culture-confirmed cases with *Salmonella* with receipt of culture or specimen between 1 January 1997 and 31 December 2005. For each case we selected 10 control persons who were alive at the day of receipt of the strain and matched for sex, age and county of residence.

Data from the National Prescription Database was available from 1 January 1995 until 31 December 2005.

Data analyses

We defined the index date as the date 21 days before the date the sample was entered in the National Registry for Enteric Pathogens. These 21 days are composed of two time intervals; we assumed that there is an average delay of 7 days between the visit to the doctor's office or admission to hospital and the date the sample is entered in the database. The other 14 days we consider being an 'acute phase' of infection. Drugs prescribed in this acute phase are likely to represent treatment of the *Salmonella* infection, in particular as early empirical treatment for diarrhoea, and therefore causes bias to the outcome:

Table 1. Definition of the groups of antimicrobials, total number of susceptibility-tested cases and prevalence of resistance to four classes of antimicrobials, Denmark 1997–2005

Groups	*Salmonella* Typhimurium	*Salmonella* Enteritidis	Other serotypes	ATC code	Antimicrobials tested for in susceptibility test
Total number of patients	4675	12151	5776		
No. susceptibility tested (%)	4534 (97.0)	4195 (34.5)	1679 (29.1)		
No. (of all cases) resistant to:					
aminoglycosides	1292	37	242	J01GB	streptomycin, gentamicin, apramycin, kanamycin, spectinomycin
amphenicols	302	1	0	J01B	chloramphenicol
extended-spectrum penicillins	1354	87	236	J01CA	ampicillin, mecillinam
fluoroquinolones	164	326	460	J01M	ciprofloxacin, nalidixic acid
other antibacterials	157	788	1	J01X	colistin, nitrofurantoin, polymyxin, fosfomycin
sulphonamides and trimethoprim	1713	57	295	J01E	sulfamethoxazole, trimethoprim
tetracyclines	1643	69	463	J01A	tetracycline
third-generation cephalosporins	7	0	4	J01DD	ceftriaxone

Breakpoints used in this study for susceptibility testing are different for each year; see DANMAP 1997–DANMAP 2005.

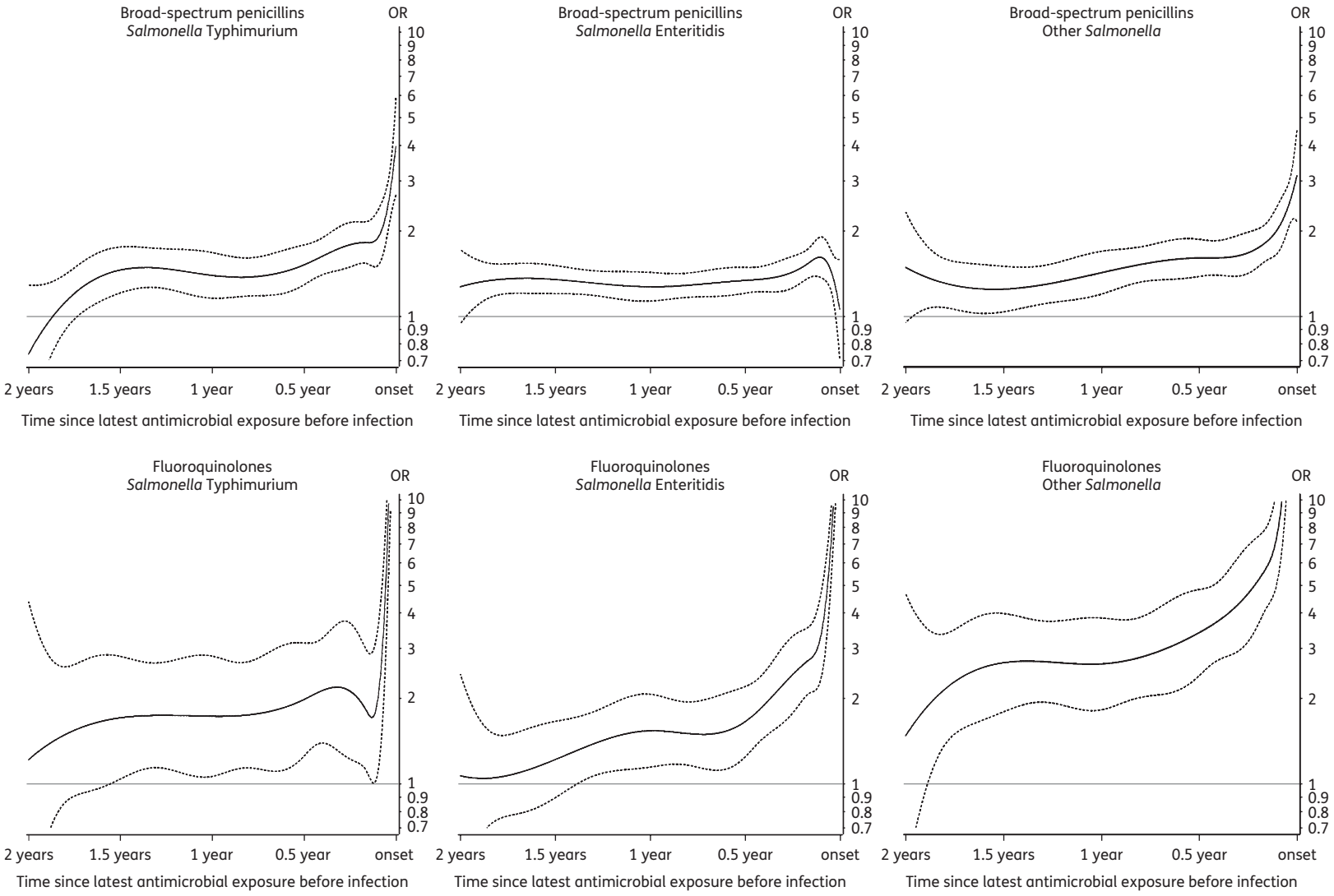

Figure 1. Cubic spline plots of the OR of being exposed to broad-spectrum penicillins and fluoroquinolones 0–2 years before infection with *Salmonella* Typhimurium, *Salmonella* Enteritidis and other *Salmonella*, 1997–2005 Denmark. The ORs are adjusted for sex, age, county of residence, population density, income and schooling.

protopathic bias.[17] For this reason, drugs taken in this time frame were excluded from our main analysis.

Odds ratios (ORs) for exposure to antimicrobial drugs before the index date were calculated in a conditional logistic regression model; Table 1 outlines the categorization of antimicrobial drugs and the number of susceptibility-tested cases. Further, a time-dependent OR for exposure to antimicrobials, in six different intervals before infection, was estimated as a function of time before onset of infection. In addition, we applied cubic splines[18] to obtain a smooth curve (Figure 1). The time frames were the 'acute phase' and 0–2 weeks, 2–4 weeks, 1–6 months, 6–12 months and 1–2 years before the index date. All estimated ORs were adjusted for rural/urban differences by population density (separated into five categories; >2000, 1001–2000, 351–1000, 26–350 and 1–25 persons/km^2), schooling (primary school only or more than primary school) and income [income per household/number of adults in the household matched within 100 000 Danish Kroner (~US $18 000) intervals].[13]

Effect modification by age was determined by fitting an interaction term by three age groups (0–15 years, 15–64 years and ≥65 years). All analyses were performed using conditional logistic regression, with the PHREG procedure in SAS 9.1.3 for UNIX (SAS Institute, Cary, NC, USA) or SAS version 9.1 for Windows.

Results

In the study period, a total of 22 602 *Salmonella* cases were reported, including 4675 (20.7%) *Salmonella* Typhimurium, 12 151 (53.8%) *Salmonella* Enteritidis and 5776 (25.6%) other *Salmonella* serotypes (Table 1). We matched a total of 214 325 controls to these patients. The median age for patients with infections of *Salmonella* Typhimurium, *Salmonella* Enteritidis and other *Salmonella* serotypes was 32, 38 and 31 years, respectively.

There was no difference in age and gender distribution among patients with a susceptibility-tested *Salmonella* strain compared with patients with strains that were not susceptibility tested. The numbers of resistant isolates were very low for some of the antimicrobials (e.g. no isolates of *Salmonella* Enteritidis were resistant to third-generation cephalosporins and no isolates belonging to the group of other serotypes were resistant to amphenicols; Table 1). Due to this limited number of observations, we excluded the following groups of antimicrobials from our analyses: amphenicols; aminoglycosides; third-generation cephalosporins; and the group named 'other antibacterials'. Either there were not enough prescriptions or the number of resistant isolates was inadequate for a meaningful analysis. Amphenicols have not been prescribed in Danish general practice since 1998, aminoglycosides and third-generation cephalosporins are mostly prescribed in hospitals and drugs that are prescribed in hospitals are not included in the National Prescription Database.

Outcome data

In the year prior to *Salmonella* diagnosis, patients more frequently had a history of antimicrobial drug use than population controls. This was observed independent of serotype and susceptibility pattern, and was found for all classes of drugs but with some variation in estimates (Table 2).

Table 2. Number of *Salmonella* cases and population controls exposed to antimicrobial drugs and the OR of *Salmonella* infection by history of antimicrobial drug use 2 weeks–12 months before infection, Denmark 1997–2005; all *Salmonella* cases, irrespective of susceptibility testing

	No. (%) exposed cases/controls		OR for being exposed to antimicrobial drugs, OR (95% CI)
	cases	controls	
Salmonella Typhimurium (4675)			
exposure to:			
broad-spectrum penicillins	601 (12.9)	3998 (9.2)	1.56 (1.41–1.73)
fluoroquinolones	78 (1.7)	326 (0.8)	2.21 (1.70–2.86)
sulphonamides and trimethoprim	177 (3.8)	1295 (3.0)	1.30 (1.10–1.54)
tetracyclines	64 (1.4)	440 (1.0)	1.32 (1.00–1.74)
Salmonella Enteritidis (12 151)			
exposure to:			
broad-spectrum penicillins	1202 (9.9)	9169 (7.8)	1.33 (1.24–1.42)
fluoroquinolones	198 (1.6)	929 (0.8)	2.07 (1.76–2.42)
sulphonamides and trimethoprim	535 (4.4)	4017 (3.4)	1.32 (1.20–1.45)
tetracyclines	194 (1.6)	1402 (1.2)	1.33 (1.14–1.55)
Other *Salmonella* serotypes (5776)			
exposure to:			
broad-spectrum penicillins	719 (12.4)	4592 (8.5)	1.62 (1.47–1.77)
fluoroquinolones	186 (3.2)	387 (0.7)	4.55 (3.78–5.47)
sulphonamides and trimethoprim	317 (5.5)	1815 (3.4)	1.72 (1.51–1.96)
tetracyclines	142 (2.5)	607 (1.1)	2.24 (1.85–2.71)

The ORs are adjusted for sex, age, county of residence, population density, income and schooling.

Table 3. Number of *Salmonella* cases and population controls exposed to antimicrobial drugs and the OR of infection by history of antimicrobial drug use 2 weeks–12 months before infection, analysed by susceptibility pattern of the infecting strain, Denmark, 1997–2005; only susceptibility-tested cases

	No. (%) exposed cases/controls		Risk for salmonellosis after exposure (main effect), OR (95% CI)	OR for resistant strain[a,b] (interaction term), OR (95% CI)
	cases	controls		
Salmonella Typhimurium (4628)				
exposure to:				
broad-spectrum penicillins	595 (12.9)	3967 (9.2)	1.50 (1.33–1.69)	1.17 (0.94–1.47)
fluoroquinolones	75 (1.6)	329 (0.8)	2.01 (1.53–2.64)	3.56 (1.22–10.34)
sulphonamides and trimethoprim	176 (3.8)	1280 (3.0)	1.33 (1.07–1.65)	0.96 (0.67–1.36)
tetracyclines	62 (1.3)	435 (1.0)	1.03 (0.70–1.51)	1.72 (0.97–3.02)
Salmonella Enteritidis (4412)				
exposure to:				
broad-spectrum penicillins	420 (9.5)	3285 (7.9)	1.26 (1.12–1.41)	1.20 (0.54–2.69)
fluoroquinolones	68 (1.5)	347 (0.8)	1.69 (1.26–2.26)	2.65 (1.20–5.85)
sulphonamides and trimethoprim	184 (4.2)	1401 (3.4)	1.26 (1.07–1.49)	2.64 (0.88–7.87)
tetracyclines	83 (1.9)	495 (1.2)	1.61 (1.26–2.05)	0.48 (0.06–3.80)
Other *Salmonella* (1619)				
exposure to:				
broad-spectrum penicillins	183 (11.3)	1224 (7.9)	1.42 (1.17–1.73)	1.53 (0.90–2.61)
fluoroquinolones	60 (3.7)	122 (0.8)	4.36 (2.97–6.41)	1.22 (0.56–2.66)
sulphonamides and trimethoprim	97 (6.0)	516 (3.3)	1.79 (1.38–2.32)	1.18 (0.60–2.32)
tetracyclines	38 (2.3)	154 (1.0)	2.30 (1.46–3.64)	1.03 (0.47–2.25)

The ORs are adjusted for sex, age, county of residence, population density, income and schooling.
[a]Cases that were exposed to antimicrobials 1 year before infection (and were infected with a strain that was resistant to the drug previously taken).
[b]Statistically this was fitted as an interaction term, the total relative risk for infection with a drug-resistant *Salmonella* that is resistant to the drug previously taken can be estimated as the product of the two ORs.

Data pertaining to susceptibility-tested strains only are given in Table 3. For several drugs (fluoroquinolones, tetracyclines and broad-spectrum penicillins), there was an effect modification, i.e. resistant strains conferred a higher OR for salmonellosis than susceptible strains. The effect modification was statistically significant for fluoroquinolone-resistant *Salmonella* Typhimurium and *Salmonella* Enteritidis. The risk of being diagnosed with a fluoroquinolone-susceptible *Salmonella* Typhimurium after having taken a course of fluoroquinolones was 2.0 times higher for patients than for controls, whereas the risk of having a fluoroquinolone-resistant *Salmonella* Typhimurium was 7.2 (2.0×3.6) times higher for patients than for controls. The logistic regression model is multiplicative, and the product in the bracket is the main effect (OR 2.0 for susceptible strains) multiplied by the interaction term (OR 3.6 for the tested strain being resistant to the antimicrobial taken). Following the same argument, for *Salmonella* Enteritidis, the OR was 1.7 for a susceptible strain whereas it was 4.5 (1.7×2.7) for acquiring a resistant strain.

We determined the OR for being diagnosed with *Salmonella* after exposure to one of the groups of antimicrobials in different time frames before infection. The outcomes of this analysis for fluoroquinolones and broad-spectrum penicillins are given in Figure 1. This graph shows the OR, on a log-scale, plotted against the time since latest antimicrobial exposure before infection with *Salmonella*, in cubic splines. In general the highest excess risk was found in a time window up to 1 month before diagnosis, and levelled out and became relatively stable 24–6 months before diagnosis.

Sulphonamides and trimethoprim were also associated with a time-dependent excess risk of being diagnosed in particular for other serotypes than *Salmonella* Enteritidis and *Salmonella* Typhimurium. The ORs were 2.14 (95% CI: 1.43–3.23) for 0–2 weeks, 1.17 (95% CI: 0.74–1.87) for 2–4 weeks, 1.23 (95% CI): 1.01–1.50) for 1–6 months, 1.27 (95% CI: 1.06–1.53) for 6–12 months and 1.40 (95% CI: 1.21–1.62) for 1–2 years before infection.

We also investigated the effect of age on the outcomes, but we did not find any significant effect modification.

Discussion

A considerable body of research has addressed the link between antimicrobial use for food animals and the selection of antimicrobial-resistant pathogens, such as zoonotic *Salmonella*, and the subsequent risk of humans acquiring such a pathogen.[7,19–23]

However, it is also known that human consumption of antimicrobial drugs is of importance. During a large outbreak of *Salmonella* Typhimurium in pasteurized milk in the USA in 1984, Ryan *et al.*[24] found that in the month before onset of illness, use of antimicrobials to which the organism was resistant increased the risk of a symptomatic infection by >5-fold.

At least two mechanisms may explain how antimicrobial drug use increases the risk of infection with resistant *Salmonella*. First, by depleting the normal gut flora and thus making the person susceptible to a smaller dose of ingested *Salmonella*. This mechanism has been described as the 'competitive effect', which is independent of the susceptibility pattern.[10,11] Secondly, by giving a selective advantage to resistant *Salmonella* strains already colonizing the gut when the antimicrobial is taken. In the study by Ryan *et al.*,[24] antimicrobial use lowered the dose of *Salmonella* needed to cause disease. The usual number of cups of milk drunk was less for ill persons who had taken antimicrobial in the month before illness than for ill persons who had not taken antimicrobials (2.4 versus 3.6 cups). This can be described as a 'selective effect', i.e. a specific advantage for the resistant pathogen.[10,11]

To our knowledge, the present study represents the first to investigate the long-term effects of antimicrobial drug exposure and, at the same time, distinguishes between these two mechanisms. The selective effect is a direct consequence of exposure to antimicrobial-resistant bacteria, in this case *Salmonella*, which has led to asymptomatic colonization. By taking a course of antimicrobials the balance of the gut flora will be disrupted, giving way to the antimicrobial-resistant bacteria to proliferate and cause clinical infection. We visualized this effect by calculating the OR that a patient was infected with a *Salmonella* strain that was resistant to the antimicrobial that was taken up to 1 year prior to the index date, of which the results are shown in Table 3. The premise of our model is that the selective effect is a multiplication of the ORs given in this table: not only should the patient be exposed to the antimicrobial, the infective strain has to be resistant to the antimicrobial exposed.

For the overall effect of being exposed to a course of antimicrobial before infection, we have taken all *Salmonella* cases into account, whether they were susceptibility tested or not. We examined whether cases or controls were more likely to have been exposed to a course of antimicrobials previous to the index date of the case. Our results indicate that a history of antimicrobial drug use may confer an increased risk of being diagnosed with *Salmonella*; this risk is present up to at least 1 year after treatment. The effect may depend on several factors including the spectrum of the antimicrobial, the antibacterial effect of its metabolites and its pharmacokinetics.

Broad-spectrum penicillins, sulphonamides and trimethoprim are excreted mainly via the urine, and therefore have a lesser impact on the gut flora.[25,26] This is also the case for most fluoroquinolones, but nevertheless, a significant part of this group of drugs is excreted in the bile. Also, some of the active metabolites of fluoroquinolones are excreted via the biliary route. This implies that fluoroquinolones and their metabolites have an impact on the gut flora by passing through the gastrointestinal tract, which may explain why the most consistent results were obtained for this drug.[27] Tetracyclines are excreted via both the biliary and the urinary pathways. This drug also reaches fairly high concentrations in the gastrointestinal tract in its unchanged form.[28]

The effect seen in the first half year before infection, in Figure 1, is a combination of the 'competitive effect', the 'selective effect' and the effect of treatment (protopathic bias). The effect of treatment can be seen if a patient had an antimicrobial prescription for diarrhoeal symptoms before the actual stool sample was taken. To account for the bias caused by the effect of treatment we introduced an index date, which excluded the 'acute phase' of exposure and an extra week that we estimated it takes for a sample to show up in the database. In Figure 1, the effect seen half a year before infection is that the OR for being diagnosed with *Salmonella* increases after exposure to a course of antimicrobials in all, except for exposure to broad-spectrum penicillins in *Salmonella* Enteritidis. We are not sure of the explanation of this effect: the half-lives of these drugs are very short (30–80 min), so it is not caused by the physical presence of the drug itself, and if that were the case, the effect should have been the same for *Salmonella* Typhimurium and *Salmonella* Enteritidis equally. The effect seen might be explained by the low percentage of resistance to this group of antimicrobials in *Salmonella* Enteritidis (~3%–6%), which is much higher in *Salmonella* Typhimurium (~44%–56%).[14]

The effect of taking a course of antibiotics 6 months to 2 years before infection can in part be ascribed to 'selection bias' (i.e. the effect that some people are more likely to be tested than other people). A history of drug use may also be an indicator of increased use of healthcare, and thus a marker of frailty, and therefore might contribute to this effect. Furthermore, a *Salmonella* carrier may develop diarrhoea due to the non-specific effect of the antibiotic and may get sampled on this basis. However, it cannot be completely ruled out that some antimicrobial drugs may have a long-term effect on the gut flora and thus render the patients more susceptible to salmonellosis.

Our study is subject to a number of limitations. One of the limitations lies within the study design, because it was an observational study and selection bias may be of importance as discussed above.

Some of the antimicrobials are mainly used in hospital settings, and these data are not included in the Danish prescription database. We can only speculate that the impact of antimicrobials on the occurrence of antimicrobial resistance would have been higher if we could also include these data. In Denmark, 90% of defined daily dosage of antibacterials is prescribed in the primary healthcare sector.[14]

We did not adjust for co-morbidity in this study, but in a similar study, performed by Gradel *et al.*,[8] this was taken into account and it did not affect the outcomes markedly. Therefore it is not likely that co-morbidity is an interaction factor in our study either.

To sum up, the use of fluoroquinolones, broad-spectrum penicillins, sulphonamides, trimethoprim and tetracyclines, up to 1 year before infection, was associated with a higher risk of *Salmonella* infection. In addition, patients who were exposed to fluoroquinolones had a significantly higher risk of contracting a *Salmonella* infection that was resistant to the antimicrobial taken, and a similar tendency was found for other classes of antimicrobial drugs.

Our data suggest that with an increasing number of antimicrobial prescriptions, the number of clinical infections with drug-resistant non-typhoid *Salmonella* is also likely to rise.

Acknowledgements

This project was presented at the ASM conference on Antimicrobial Resistance in Zoonotic Bacteria and Foodborne Pathogens, Copenhagen, June 2008 (Poster C135).

Funding

This work was supported by the EU Marie Curie Programme (MEST-CT-2004-007819) and the Danish Food Industry Agency (grant number 3304-FVFP-07-721-01).

Transparency declarations

No conflicts of interest to report.

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Manuscript II:

Antimicrobial Use: A Risk Factor or a Protective Factor for Acquiring Campylobacteriosis?

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MAJOR ARTICLE

Antimicrobial Use: A Risk Factor or a Protective Factor for Acquiring Campylobacteriosis?

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Background. It is well acknowledged that the use of antimicrobial drugs in food animals leads to antimicrobial drug resistance in foodborne bacteria such as *Campylobacter*; however, the role of human antimicrobial usage is much less investigated. The aim of this study was to quantify the odds of campylobacteriosis conferred by human consumption of fluoroquinolones and macrolides.

Methods. We conducted a registry-based retrospective case-control study on 31 669 laboratory-confirmed cases of campylobacteriosis between 1999 and 2005 in Denmark. Data were obtained from several Danish databases: the National Registry of Enteric Pathogens, the Danish Civil Registration System, the Danish National Prescription Database, and the Integrated Database on Labor Market Research. Odds ratios (OR) for campylobacteriosis were calculated by conditional logistic regression.

Results. The risk of campylobacteriosis was reduced 1 month after exposure to macrolides (OR, 0.72; 95% confidence interval [CI], 0.56–0.92). Macrolide exposure 1 month to 2 years before infection was associated with an increased risk of a *Campylobacter* diagnosis (OR, 1.5; 95% CI, 1.4–1.6). A history of fluoroquinolone use was also associated with increased risk (OR, 2.5; 95% CI, 1.8–3.5). This risk was higher for resistant isolates than for susceptible ones.

Conclusions. Treatment with macrolides may protect against *Campylobacter* infection for a limited period of time, possibly due to the antibacterial effects of the drug or its metabolites. Fluoroquinolone treatment confers increased risk, probably due to a combination of competitive and selective effects, similar to what has been observed for nontyphoid *Salmonella* infection.

Campylobacter jejuni is recognized worldwide as the leading cause of bacterial gastroenteritis [1]. In 2008, a total of 190566 campylobacteriosis cases were reported (overall incidence of 40.7 per 100 000) by 25 EU Member States [2]. The level of antimicrobial resistance of this bacterium has been rising for many years. Resistance against fluoroquinolones and macrolides is of particular concern for public health. A major source of human campylobacteriosis is poultry and products hereof, and several studies indicate that use of antimicrobials such as fluoroquinolones and macrolides in poultry production selects for drug-resistant *Campylobacter* [3–5]. Drug-resistant *Campylobacter* transferred to humans in the food chain may cause more severe illness than sensitive strains [6]. Less well understood and described is the role of human consumption of antimicrobial drugs. Only a few studies highlight the role of this consumption on the development of antimicrobial drug resistance in *Campylobacter* [7–9]. The vast majority of publications focus instead on the role of other risk factors, like travel association, consumption of chicken and other food products, contact with animals, and other factors [10–12].

In the present registry-based study, we evaluated the association between fluoroquinolones and macrolides prescribed in general practice on the occurrence of subsequent infection. We also estimated the odds of diagnosis with a resistant strain after exposure to a course of antimicrobials; we accomplished this by

Received 21 December 2010; accepted 10 May 2011.

Presented in part: 5th Annual Scientific Meeting Med-Vet-Net, El Escorial, Spain, June 2009.

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Clinical Infectious Diseases 2011;53(7):644–650

1058-4838/2011/537-0004$14.00
DOI: 10.1093/cid/cir504

fitting an interaction term into the model between the odds of being diagnosed with campylobacteriosis and the odds of being diagnosed with a resistant strain.

METHODS

Registers

All culture-confirmed cases of *Campylobacter* are reported to the Statens Serum Institut (SSI) and entered into the National Registry for Enteric Pathogens [13]. This register only includes each patient's first isolate within a time frame of 6 months; if the patient has another positive sample within this period, it is considered to be a recurrent or persistent infection and is discarded in the database. During the study period (1999–2005) 32.4%, of 31 669 isolates reported to SSI were susceptibility tested against fluoroquinolones and macrolides. For these strains, we calculated excess odds of campylobacteriosis after drug exposure, as well as a multiplicative interaction term (the odds that the infective strain was resistant to the antimicrobial previously taken). For a more comprehensive testing of resistance, a total of 459 strains were tested for a broader panel of antimicrobial drugs (Table 1) as a part of the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) surveillance scheme [16]. However, due to the low number of strains examined, we did not have statistical power to calculate the interaction term for these antimicrobials. The data were linked using the unique, personal identification number used in the Civil Registry System (CRS) [17]. Information on county of residence can directly be deduced from this database.

To obtain data on exposure to antimicrobial drugs prior to the *Campylobacter* infection, we used the National Prescription Database. This database contains information on all prescriptions provided by general practitioners, which were filled at pharmacies in Denmark. Data were once again linked using the Civil Registry System number and included the generic name of the drug, the ATC-code, dosage, price, and date of issue. To control for the confounding effect of education and socioeconomic status, we obtained data on schooling and income from the Integrated Database on Labor Market Research [18].

Study Participation

We included all laboratory-confirmed patients with campylobacteriosis between 1 January 1999 and 31 December 2005. For each case, we selected 10 controls from the Civil Registry System who were alive on the date the positive sample was received at the SSI and matched them to the case on sex, age, and county of residence. Data from the prescription database were available from 1 January 1995 until 31 December 2005.

We compared the history of antimicrobial use in *Campylobacter* patients to the history of use of their matched controls in a period up to 2 years prior to diagnosis.

Susceptibility Testing

The antimicrobial drugs of interest for this study were fluoroquinolones and macrolides. To simplify the analysis, strains that were resistant against nalidixic acid and/or ciprofloxacin were considered resistant against fluoroquinolones; strains that were resistant against erythromycin were considered resistant against macrolides. All strains that were typed ''intermediate'' resistant were considered to be susceptible. All antimicrobial susceptibility testing was performed with a commercially available MIC technique (Sensititre, Trek Diagnostic Systems Ltd). The cells were inoculated and incubated according to the CLSI guidelines. The breakpoints for this testing have changed over the years and can be found in the annual DANMAP reports (www.danmap.dk) [19].

Data Analyses

We conducted a registry-based, matched, retrospective case-control study. For this purpose, we defined an index date as the date 21 days before the date when the sample was entered

Table 1. Definition of the Groups of Antimicrobials Used in This Study

	ATC code	Antimicrobials susceptibility tested
Antimicrobials for which the excess risk of campylobacteriosis and the interaction term was calculated		
Fluoroquinolones	J01M	Ciprofloxacin, nalidixic acid
Macrolides	J01F	Erythromycin
Antimicrobials for which only the excess risk of campylobacteriosis was calculated		
Aminoglycosides	J01GB	Streptomycin, gentamicin, apramycin, kanamycin, spectinomycin
Amphenicols	J01B	Chloramphenicol
Other antibacterials	J01X	Nitrofurantoin, polymyxin
Sulfonamids and Trimethoprim	J01E	Sulfamethoxazole, trimethoprim
Tetracyclines	J01A	Tetracycline
Third generation cephalosporins	J01DD	Ceftriaxon

Breakpoints used in this study for susceptibility testing are different per year: see DANMAP1997 [14] and —DANMAP2005 [15].

in the National Registry for Enteric Pathogens. These 21 days are composed of 2 time-intervals: first, a 7-day average between the visit to the general practitioner's office or admission to hospital, and the date the sample was entered in the database; and second, the 14 days we consider the ''acute phase'' of infection. Antimicrobial drugs prescribed in this acute phase are likely to be prescribed for early empirical treatment of the *Campylobacter* infection, and therefore causes protopathic bias [20]. Drugs taken in this time frame were excluded from the main analysis but were included in an additional subanalysis described later.

In the main part of the analysis, we examined the odds of exposure to a course of antimicrobials before being diagnosed with *Campylobacter*, from the index date to 1 year before onset of disease. We also calculated the odds of a strain being resistant against the antimicrobial taken. Odds ratios (ORs) for exposure to antimicrobial drugs before infection were calculated using a conditional logistic regression model; Table 1 outlines the categorization of antimicrobial drugs.

In the second part of the study, we calculated a time-dependent OR for exposure to antimicrobials in 6 different time intervals before infection. The OR was estimated as a function of time before onset of infection. In addition, we applied cubic splines [21] to obtain a smooth curve (Figure 1). The coefficients for the cubic spline function were estimated in a conditional logistic regression model. In the cubic spline model 3 knot points were used; these were chosen to be at 60 d, a half year, and 1 year before onset of infection. A knot point is a point where the coefficients for the third-degree polynomial were allowed to change, although only under the restriction that the curve has to be continuous and smooth in the knot points.

The time frames were the ''acute phase,'' 0–2 weeks, 2–4 weeks, 1–6 months, 6–12 months, and 1–2 years before index date. All of the estimated ORs were adjusted for rural/urban differences by population density (separated in 5 categories: >2000, 1001–2000, 351–1000, 26–350, 1–25 persons per square kilometer), schooling (primary school or more than primary school), and income (income per household/number of adults in the household matched within 100 000 dkk (~19 000 USD) intervals) [13].

All analyses were performed using conditional logistic regression with the PROC PHREG- procedure in SAS 9.1 for Windows (SAS Institute).

RESULTS

In the study period, a total of 31 669 *Campylobacter* cases were reported, of which 10 275 were susceptibility tested for both fluoroquinolones and macrolides. These 10,275 patients were matched (1:10) to 97 523 controls. Overall, 21.7% was resistant against fluoroquinolones and 2.3% against macrolides (Table 2). The prevalence of fluoroquinolone resistance was highest in adults 50–59 years of age and lowest in children. A smaller sample was additionally tested for susceptibility to aminoglycosides, amphenicols, other antibacterials (ATC-code J01X), sulfonamids and trimethoprim, tetracyclines, and third-generation cephalosporins.

Being diagnosed with campylobacteriosis was associated with an increased odds of exposure to a course of fluoroquinolones, macrolides, sulfonamids and trimethoprim, tetracyclines, and broad spectrum penicillins up to 1 year before onset of disease (Table 3). This risk was highest for taking fluoroquinolones (OR, 2.4; 95% CI, 1.97–2.97). For fluoroquinolones, we found an effect modification, that is, resistant strains conferred a higher risk of diagnosis than susceptible strains. The odds of being exposed to a course of fluoroquinolones was 2.4 times higher for cases diagnosed with a fluoroquinolone-sensitive *Campylobacter* than for controls, whereas the odds of being exposed to fluoroquinolones was 3.8 (2.4 × 1.6) times higher for cases with a fluoroquinolone-resistant *Campylobacter* than for controls. The logistic regression model is multiplicative, and the product in the brackets is the main effect (OR 2.4 for susceptible strains) multiplied by the interaction term (OR 1.6 for the tested strain being resistant to the antimicrobial taken). There was no interaction between macrolide exposure and macrolide resistance.

In the second analysis of this study, we calculated the risk (OR) of previous exposure to antimicrobials in different time intervals for people who had been diagnosed with *Campylobacter*. The results of this analysis are given in Figure 1. This graph shows the OR, plotted against the time since latest antimicrobial exposure before infection with *Campylobacter*, in cubic splines. The results for fluoroquinolones and macrolides were different. Diagnosis with *Campylobacter* was associated with a decreased odds of macrolide consumption in the period close to diagnosis, while it was associated with an increased fluoroquinolone consumption in the same period (Table 4). We examined the effect of macrolides more thoroughly to determine the duration of this protective effect and performed the analyses for 4 different macrolides separately; erythromycin, roxithromycin, clarithromycin, and azithromycin. There was an excess odds of consumption of each of these drugs up to 1 year before infection (Table 4). However, if the time between last exposure and *Campylobacter* infection is reduced to only the acute phase of infection (the estimated time before onset of disease and appearance in our database), exposure to macrolides became a protective factor (OR .72, up to 1 month before infection; Table 4).

DISCUSSION

We have recently shown that a history of use of several different classes of drugs is associated with an excess risk of being diagnosed with nontyphoid *Salmonella* [22]. Furthermore, this

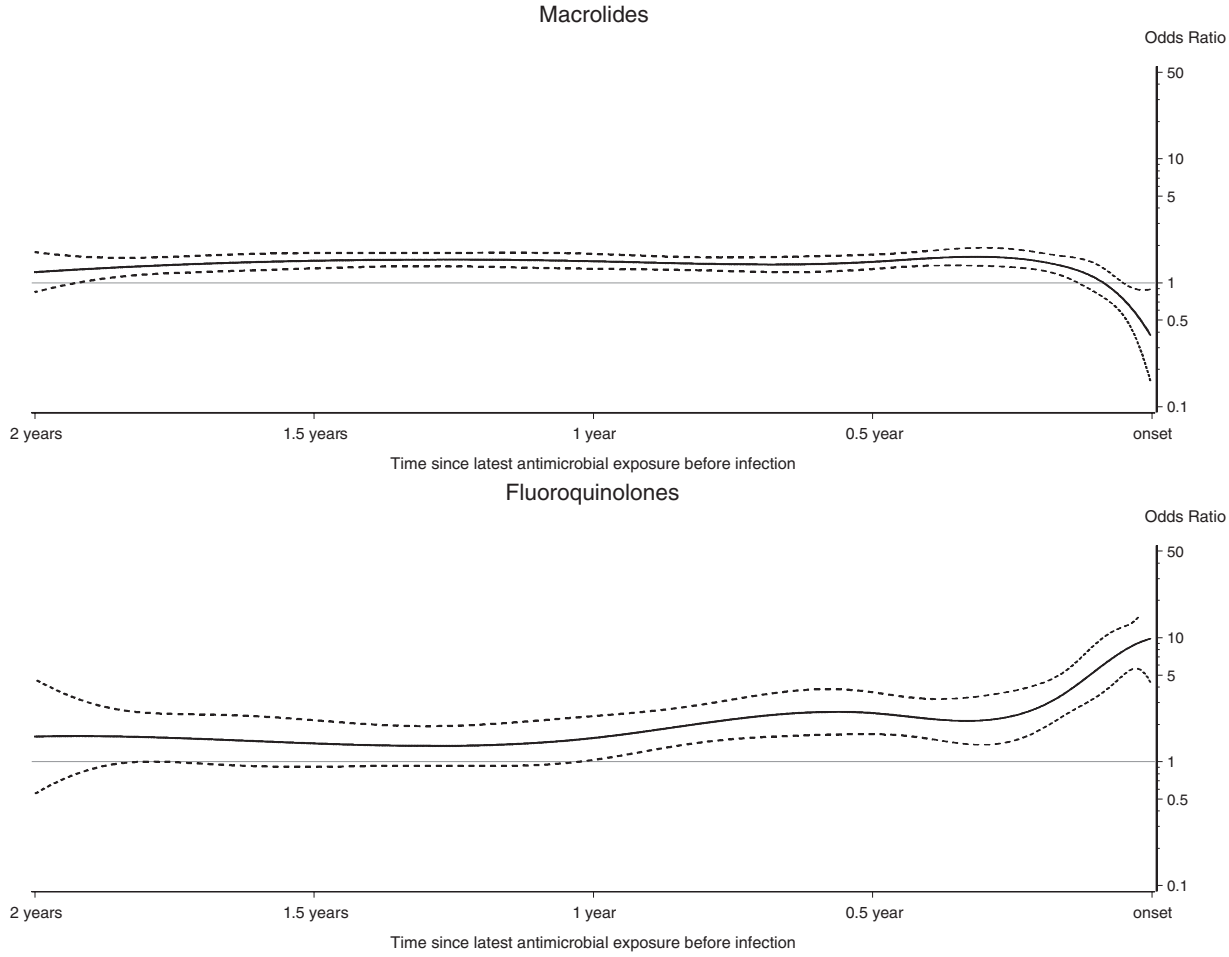

Figure 1. Cubic spline plots of the odds ratio (OR) of being exposed to macrolides and fluoroquinolones 0–2 years before infection with *Campylobacter*, 1997–2005 Denmark. The ORs are adjusted for sex, age, county of residence, population density, income, and schooling..

Table 2. Age Distribution and Prevalence of Resistance in 10475 *Campylobacter* Strains Examined for Susceptibility for Fluoroquinolones and Macrolides, Denmark, 1999–2005

Age groups	Total no. of patients N (%)	No. and % susceptibility tested[a] N (%)	Fluoroquinolone resistance[b] N (%)	Macrolide resistance[c] N (%)
0–9 years	4076 (14.9)	1556 (38.2)	224 (14.4)	25 (1.6)
10–19 years	3039 (11.1)	1150 (37.8)	231 (20.1)	21 (1.8)
20–29 years	6841 (25.1)	2750 (40.2)	613 (22.3)	57 (2.1)
30–39 years	4971 (18.2)	1958 (39.4)	428 (21.9)	40 (2.0)
40–49 years	3117 (11.4)	1098 (35.2)	281 (25.6)	32 (2.9)
50–59 years	2505 (9.2)	992 (39.6)	256 (25.8)	32 (3.2)
60–69 years	1436 (5.2)	542 (37.7)	137 (25.3)	21 (3.9)
70–79 years	835 (3.1)	293 (35.1)	72 (24.6)	8 (2.7)
80 and older	456 (1.7)	136 (29.8)	26 (19.1)	2 (1.5)
Total	27,276 (100)	10,475 (38.4)	2268 (21.7)	238 (2.3)

[a] Overall χ^2: 45.0, degrees of freedom: 8, *P* value: $< .001$.

[b] Overall χ^2: 69.8, degrees of freedom: 8, *P* value: $<.001$.

[c] Overall χ^2: 14.8, degrees of freedom: 8, *P* value: .06.

Table 3. Risk of *Campylobacter* Diagnosis by Exposure to a Course of Antimicrobial Drugs 0–12 Months Before Infection, Denmark, 1999–2005

Exposed to (up to 1 year before infection):	No. (%) exposed cases/controls		Risk for campylobacteriosis after exposure (main effect) OR (95% CI)	OR for resistant strain [a,b] (interaction term) OR (95% CI)
	Cases (10275)	Controls (97591)		
Broad-spectrum penicillins	1191 (11.6)	8756 (9.0))	1.35 (1.25, 1.45) $P \leq .001$	
Fluoroquinolones	183 (1.8)	658 (0.7)	2.42 (1.96, 2.98) $P \leq .001$	1.61 (1.11, 2.32) $P = .01$
Sulfanomids and Trimethoprim	530 (5.1)	3535 (3.6)	1.48 (1.34,1.64) $P \leq .001$	
Tetracyclines	202 (2.0)	1437 (1.5)	1.36 (1.16, 1.59) $P \leq .001$	
Macrolides	1158 (11.3)	7642 (7.8)	1.48 (1.38, 1.59) $P \leq .001$	1.04 (0.70, 1.54) $P = .85$

The odds ratios (ORs) were adjusted for sex, age, county of residence, level of schooling, income, and population density.

[a] Cases that were exposed to antimicrobials 1 year before infection (and were infected with a strain that was resistant to the drug previously taken).

[b] Statistically, this was fitted as an interaction term, the total relative risk for infection with a drug-resistant *Campylobacter* that is resistant to the drug previously taken can be estimated as the product of the 2 ORs.

effect was substantially modified by the resistance pattern of the infecting strain [22]. This finding strongly supports the idea of competitive and selective effects of antimicrobial drug use [23, 24]. The results of the present study are in line with the fluoroquinolone findings in our *Salmonella* study, as well as our observation that a history of human antimicrobial-drug exposure may constitute a long-term risk for being diagnosed with *Campylobacter*. Surprisingly, in the current study, diagnosis with *Campylobacter* was associated with a reduced odds of recent history of macrolides compared with controls. This raises the hypothesis that macrolide use may protect against *Campylobacter* infection for a limited period of time, perhaps as long as 4–8 weeks (Table 4).

To assess this hypothesis, different effects of antimicrobial drugs should be considered, including their spectrum, the antibacterial effect of their metabolites, and their pharmacokinetics. The pharmacokinetics of macrolides are well described in the literature; the half-life of macrolides is different for each of the drugs included in this study; erythromycin has a short half-life (<4 hours), roxithromycin and clarithromycin an intermediate half life (4–24 hours) and azithromycin has a long half-life (>72 hours) [25]. However, the protective effect of the drugs last at least 4 weeks (Figure 1). The effect of macrolides, as a group, in the acute phase and 0–2 weeks before the index date (OR, .72; 95% CI, .56, .92) is strong. The acute phase was specifically constructed so we could not confuse drugs prescribed as early empirical treatment as a risk factor for acquiring *Campylobacter*.

It is interesting that a protective effect is seen in all the macrolides (Table 4), even though the kinetics of each of the macrolides is quite different. One explanation for this phenomenon is that macrolides, especially azithromycin, become trapped in the intracellular lysosomes of phagocytic cells. This intracellular uptake is easily reversible for erythromycin and clarithromycin but is extremely slow and probably not completely reversible for azithromycin [26, 27]. It is even possible

Table 4. Risk of *Campylobacter* Diagnosis by Timing of Exposure to a Course of Antimicrobials 0–12 Months Before Infection, Denmark, 1999–2005

Exposed to:	Upto 1 year before infection:[a] OR (95% CI)	Upto 3 months before infection[b]: OR (95% CI)	Upto 2 months before infection[b]: OR (95% CI)	Upto 1 month before infection[b]: OR (95% CI)	Only the acute phase: OR (95% CI)
Erythromycin	1.40 (1.25–1.57) $P \leq .001$	*Not enough power for the separated analyses*			0.06 (0.01–0.43) $P = .005$
Roxithromycin	1.63 (1.37–1.93) $P \leq .001$				0.39 (0.09–1.63) $P = .20$
Clarithromycin	1.62 (1.29–2.02) $P \leq .001$				0.83 (0.25–2.71) $P = .75$
Azithromycin	1.44 (1.30–1.60) $P \leq .001$				0.29 (0.11–0.79) $P = .02$
Macrolides grouped	1.48 (1.38–1.59) $P \leq .001$	1.16 (1.02–1.31) $P = .02$	0.98 (0.83–1.16) $P = .81$	0.72 (0.56–0.92) $P = .01$	0.30 (0.16–0.56) $P = .01$
Fluoroquinolones	2.42 (1.96–2.98) $P \leq .001$	2.54 (1.83–3.54) $P \leq .0001$	2.65 (1.78–3.93) $P \leq .0001$	2.94 (1.82–4.75) $P \leq .0001$	10.32 (6.65–16.02) $P = .002$

The odds ratios (ORs) were adjusted for sex, age county of residence, level of schooling , income, and population density.

[a] This period does not include the ''acute phase'' of infection.

[b] These periods do include the ''acute phase'' of infection.

that part of the azithromycin metabolites remain trapped in the lysosome until the cell dies, and the drug is finally released (N. Frimodt-Møller, personal communication, 2009).

Antimicrobial drug promotors, given to food animals in subinhibitory concentrations, may have profound effects on the intestinal flora and risk of infection with gastrointestinal infections. It cannot be ruled out that macrolides or their metabolites are present in intestines of humans for several weeks in sufficiently high concentrations to produce a similar effect. This may be the case for azithromycin, as well as other drugs. Clearly, additional studies are needed to examine this possibility.

Other explanations for our observation should be considered. Although macrolides are mostly prescribed in the winter season for respiratory infections, and *Campylobacter* infections are most common in the late summer [28, 29], confounding effects by season are not a likely explanation for the association because we adjusted for season by matching on the index date of the case. Furthermore, several other classes of antimicrobial drugs are prescribed more commonly in the winter season as well, without causing a similar pattern [22].

For fluoroquinolones, we found a risk pattern similar to the risk pattern found when examining their effects with nontyphoid *Salmonella* infection [22]. The observation, as shown in Figure 1, can be ascribed to a combination of 3 effects: the "competitive effect," the "selective effect," and for fluoroquinolones, also the effect of treatment (protopathic bias). The effect of treatment can be seen in the 'acute phase' of the infection where the patient might have received a course of fluoroquinolones as early empirical treatment, before the stool-sample was taken. The competitive effect is independent of the susceptibility pattern and can be explained by depletion of the normal gut-flora, thereby leaving the person susceptible to a smaller dose of an infective agent, in this case *Campylobacter*. Finally, the selective effect is the advantage to resistant *Campylobacter* strains already colonizing the gut when the antimicrobial was taken [23, 24].

We assume that the observed effect in Figure 1, from a point where the line stabilizes (at ~3 months before infection for macrolides and a year for the fluoroquinolones) can be considered as a base-line for "selection bias" (the effect that some people are more likely to be tested than other people). A history of drug use may also be an indicator of increased use of health care (and thus a marker of frailty) and therefore might contribute to this effect [30]. Furthermore, an asymptomatic *Campylobacter* carrier may develop diarrhea due to the nonspecific effect of the antibiotic and may be sampled based on this. However, it cannot be completely ruled out that some antimicrobial drugs may have a long-term effect on the gut flora and thus render patients more susceptible to campylobacteriosis.

We found some differences in the number of samples susceptibility-tested in each age-group (Table 2). Even though the differences are significant, we assume that this is due to random error. We also found a significant difference in the number and percentages of fluoroquinolone-resistant individuals for different age groups. This effect is likely to be explained by 2 factors: (1) the fact that it is not common to prescribe fluoroquinolones to children and (2) the differences in travel behavior between the age groups. In 2008, 27.4% of *Campylobacter* cases were travel related [31] and were associated with a higher percentage of resistance [16]. However, matching for age should have eliminated any related bias.

The study is subject to some limitations. First, some antimicrobial drugs are mainly used in hospital settings, and these data are not included in the Danish prescription database. However, in Denmark, 90% of the prescribed antimicrobials are prescribed in the primary health sector [16]. We can only speculate that the impact of these antimicrobials would have been larger if they were included in the study.

Second, it is well known that *Campylobacter* shows immense antigenic diversity, and immune-evasion strategies can differ greatly between different strains of *Campylobacter* [32]. Unfortunately, species identification and subtyping of *Campylobacter* is not carried out as a routine activity in Denmark; therefore, these data were not available for use.

To conclude, human use of broad-spectrum penicillins, sulfonamids, trimethoprim, tetracyclines, macrolides, and in particular fluoroquinolones may form a risk factor for acquiring campylobacteriosis. Furthermore, fluoroquinolone consumption selects for infection with resistant *Campylobacter*. To our surprise, *Campylobacter* diagnosis was associated with a reduced odds of recent macrolide use.

Notes

Financial support. This work was supported by the Danish Food Industry Agency (grant 3304-FVFP-07-721-01).

Potential conflicts of interest. M. K. received funding through his institution from Med-Vet-Net for attending the Med-Vet-Net conference where the study results were presented in an oral presentation. All other authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Manuscript III:

History of antimicrobial treatment and severity of *Salmonella* Typhimurium infections

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History of antimicrobial treatment and severity of *Salmonella* Typhimurium infections

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Running title: Severity of *Salmonella* Typhimurium infections

Summary (150 words)

We performed a prospective case-case telephone-interview study in Denmark, including 150 *Salmonella* Typhimurium patients with the aim to examine how clinical outcome of an infection is affected by previous antimicrobial exposure. In addition, we were interested in differences in outcome of disease relating to the susceptibility profile (fully susceptible, resistant and multiresistant). We found that previous antimicrobial use, unrelated to this salmonellosis was related to a higher risk of weight loss (OR 2.43 95%CI 1.09-5.48), hospital admission (OR 2.03 95%CI 1.02-4.06), or antimicrobial therapy for the current salmonellosis (OR 7.85 95%CI 2.77-16.78). We were not able to demonstrate major negative outcomes of infection with antimicrobial drug resistant *Salmonella* Typhimurium infections in connection with the three different levels of drug-resistance. The higher risk of weight loss, hospital admission or therapy among patients with a history of antimicrobial treatment independent of the current episode adds to studies that have demonstrated competitive and selective effects of antibiotic treatment.

Introduction

Salmonella Typhimurium is among other non-typhoid *Salmonella* infections one of the most common causes of foodborne bacterial gastrointestinal infection worldwide, usually causing a self-limiting diarrhoeal disease, but occasionally causing a more severe disease outcome, such as reactive arthritis, bacteraemia or even death, particularly in very young children or the elderly.(1) Antimicrobial drugs are not recommended as a routine in the management of infections with non-typhoid *Salmonella* infections, however when severe illness or complications are suspected, or infections in patients with underlying illness occur, antimicrobials are often prescribed. The effect of early empirical treatment is under threat due to increasing antimicrobial drug resistance which has been observed in *Salmonella*, not only worldwide, but even in Denmark where antimicrobial use in the food industry has been much lower than in the rest of the world.(2-4) A consequence of increasing antimicrobial resistance may be that appropriate treatment for salmonellosis can be delayed, which could lead to more serious outcome of disease.(5;6)

Several host-associated risk factors and high-risk food items for salmonellosis have been identified from case-control studies of sporadic disease and outbreak investigations. Besides common sources including pork, eggs and chicken, *Salmonella* Typhimurium outbreaks have been associated with vehicles such as instant baby-formula powder, cheese consumption, dry dog- and cat-food, to mention a few of the diverse food items incriminated in outbreaks.(7-12) Infections may often be acquired during foreign travel. Host-associated risk factors include old age or being an infant(13;14) as well as presence of underlying (chronic) disease, these factors are described as determinants for a more severe outcome for salmonellosis. Another predictor for a more severe outcome of disease is being infected with an antimicrobial resistant *Salmonella* (15-21), hardly any studies show the opposite(22).

In a registry based study, we have showed that previous exposure to antimicrobial drugs is an important risk factor for infection with a non-typhoid *Salmonella*. In the present prospective case-case comparison we wanted to examine further how antimicrobial exposure before onset of *Salmonella* infection may affect outcome of disease. In addition, we wanted to analyse whether *Salmonella* Typhimurium infections with different susceptibility profiles (fully susceptible, resistant, and multiresistant) were associated with different outcomes of disease.

Materials and Methods

Study design and definitions

This study was designed as a prospective case-case interview study, including patients with acute gastroenteritis or invasive infection due to *Salmonella* Typhimurium; for the purpose of the present study monophasic *Salmonella* (*S.*4,5,12,I-) was included as well. All patients will be referred to as *Salmonella* patients. The patients were split into three groups, according to the antimicrobial susceptibility profile of the *Salmonella* strain that they were infected with. The first group had a fully susceptible profile (S), the second group was resistant against one to three different antimicrobial classes (R), and the last group was multiresistant (MR), i.e. resistant to four or more antimicrobial drugs from different classes. MIC testing is used to determine antimicrobial resistance in the laboratory, isolates are tested susceptible (S), resistant (R), or intermediate resistant (I) for antimicrobial drugs, according to EUCAST standards (www.eucast.com).(23) If the MIC-value of the isolate was tested I to a drug, it was coded as S. In Denmark, *Salmonella* are tested for 19 antimicrobials: ampicillin, apramycin, amoxicillin-clavulanic acid, cefotaxime, ceftiofur, chloramphenicol, ciprofloxacin, collistin, florfenicol, fosfomycin, gentamicin, mecillinam, nalidixic acid, neomycin, spectinomycin, streptomycin, sulfonamide, tetracycline and trimethoprim.

Identification of patients

Criteria for enrolment in the study were: confirmation either by means of faecal culture or by a culture from a normal sterile site, that were reported to the Statens Serum Insitut (SSI), and entered into the National Registry for Enteric Patients(24), and being reported between January and June 2010. Susceptibility testing by the Unit of Gastrointestinal Infections of SSI(25) is generally performed on nearly all (i.e., 94% in the study period) strains submitted for subtyping.(26) All Danish citizens can be tracked in the health care system and national registries using the unique ten digit civil registration number assigned to each person at birth or upon immigration.(27) If several persons from the same household were reported within the same 2 weeks, only the first to be registered was interviewed.

Patients belonging to outbreak clusters were excluded from the study. Patients were also excluded if they had travelled outside of Denmark in the two-week period before onset of first symptoms.

Interview and questions

All *Salmonella* patients were interviewed by an interview-team at SSI using a structured questionnaire, see Appendix.

The interview was performed within a month of the date that the sample was submitted in the National Register for Enteric Patients. The questionnaire contained 22 questions concerning outcome of disease, duration of symptoms, severity of disease, and patient characteristics. The variables included in the study can be found in Tables 1 and 3. For children under the age of 15 years, a parent or a guardian was interviewed. For children between 15 and 17 years of age, we first asked the parents or guardian to give their consent to have their children interviewed before approaching the patient.

Data analysis

Data handling

For the continuous variable age, we created categories in years, 0-3 'infants', 4-17 'children', 18-64 'adults', 65 and older 'seniors'. Underlying comorbidity was added in the analysis as a 0/1 variable; consisting of having any of the following underlying illness: asthma/bronchitis, heart and circulation disease, intestinal illness, recurrent diarrhoea, liver disease, diabetes, connective tissue disease, kidney problems, cancer, chronic infection, other disease.

Analyses

We analysed the data in three separate groups according to resistance profile: S, R and MR. Statistical tests of associations between resistance profile and disease outcome were computed by use of χ^2-test and Fisher's exact tests, as well as likelihood ratio tests in a logistic regression model. We used a significance α–level of 5% for these analyses. We only checked for biologically reasonable confounding and interaction factors, like age, level of schooling, income, smoking, and self-reported stress. Confounding and interactions were tested for in a multivariable regression model.

Data handling

Defgo.net® software was used to keep the structured interview and storage of the outcomes. All analyses were performed with SAS software version 9.3.

Results

Interview results

In total, 228 patients were available during our study period and 150 (66%) were interviewed. The remaining 78 patients could not, or did not want to be interviewed for various reasons, see Figure 1 for a flow chart. The ages of the people who died before they could be interviewed were 56, 63, 72 and 74 years of age, the cause of death was not known to the researchers.

Patient characteristics

Table 1 shows the age and gender distribution and variables for the severity of the disease relating to all 150 patients. The median age was 36 years (interquartile range: 10-57 years). Twenty-six (17%) of these patients were under four years of age, and 24 (16%) were 65 years or older. Common symptoms included: abdominal pain (>7 days, 81%), fever (>7 days, 73%), and severe diarrhoea (>7 days, 69%). The median duration of diarrhoea was 13 days (interquartile range 7-15 days). Forty-nine patients (33%) were hospitalised for their *Salmonella* infection, and 76 (51%) of the patients received antimicrobial treatment for the current infection. Among 24 patients aged 65 or older, 12 (50%) had been admitted to hospital, and 18 (75.0%) had received antimicrobial treatment for the current infection.

Most patients (90, i.e. 60%), had no underlying disease, among those with underlying disease, asthma was the most frequent condition, (21 patients; 14%), followed by cardiovascular disease (12 patients, 8%), connective tissue disease (nine patients, including patients with rheumatoid disease), and diabetes (eight patients). In total, 27 (45%) of patients with underlying illness were treated with antimicrobial drugs compared with 41 (46%) among those with no underlying illness.

Resistance pattern and phage types

Of all 150 patients, 49 (33%) had a fully susceptible resistance pattern (S), 48 (32%) had a resistant resistance pattern (R), and 53 (35%) patients had a multidrug-resistant pattern (MR). Only two cases were resistant to nalidixic acid, a marker for resistance against the quinolones.

Comorbidity was more common in patients with a resistant strain (21 of 48 patients, 44%) or multidrug-resistant strain (27 of 53, 51%) compared with susceptible strains (12 of 49, 24%), Table 1. This pattern was partly related to age differences since younger patients had less comorbidity and were more likely to have a susceptible strain. There were more children under four years of age in the S group than in the R or MR groups (OR 3.09 (95%CI 1.08 – 8.82) and OR 4.24 (95%CI1.41 – 12.77), respectively). We included both age and resistance profile to be explanatory variables for comorbidity, see Table 2.

Only few clinical outcomes were dependent on the resistance profiles, and all of those were between the R group compared to the S group. For the R group the odds of being hospitalised, vomiting or experience nausea for more than 7 days were higher: OR 2.47 (95%CI 1.02 – 5.97), OR 2.61 (95%CI 1.10 – 6.19), and OR 2.87 (95%CI 1.26 – 6.54), respectively.

Different phage types were found in the different groups of patients. The most common phage types found in the MR group were DT193 and DT120 with each 11 patients (20.8%), for the R group the most pathogens were classified as React but Did NOT Confirm (RDNC) (N=30, 62.5%) and DT120 (N=7, 14.6%), and for S group it were U292 (N=15, 30.6%) and DT135 (N=10, 20.4%).

Severity of outcome in relation to antimicrobial use and age

In total, 68 patients (45%) stated that they had a history of antimicrobial treatment in the six months period preceding infection. Patients with a history of treatment tended to have a higher risk of infection with resistant or multidrug-resistant *Salmonella* strains, but these differences were not significant (Table 1). Furthermore, a simple analysis, combining resistant with multiresistant strains into one group, did not reach statistical significance (OR 1.31; 95% CI 0.62-2.79). However, patients who had been exposed to a course of antimicrobials, not related to the *Salmonella* infection, up to half a year before they got infected with *Salmonella*, had a higher odds of being hospitalised, losing more than 5 kg weight and receiving treatment for the current event of salmonellosis (Table 3).

Discussion

The main outcomes of this study were that young children have higher odds for being infected with a fully susceptible strain of *Salmonella* and are less likely to have underlying diseases, and older people have higher odds for being infected with a multiresistant strain of *Salmonella* and also have higher odds of having an underlying comorbidity. In addition, a history of antimicrobial drug use was related to severe outcome of infection including need for treatment, hospital admission and weight loss. This observation adds to the literature on the public health consequences of use of antimicrobials. It is possible that human antimicrobial treatment disrupts the normal intestinal flora and can have long term negative effects for human health.(28;29)

It should, however, be mentioned that because patients are treated for an infection, and that part of the association may be attributed to confounding by indication, which should be investigated in future studies. Furthermore, it can be speculated that people with underlying illnesses are more vulnerable and are therefore more likely to have been exposed to antimicrobials and consequently more likely to be infected with an antimicrobial resistant strain. These findings would be in concordance with our previous studies(39)

We found that about one of two patients was treated with antimicrobial drugs. This was a surprise for us since Denmark is known for a prudent use of antibiotics. Elderly patients, over 60 years, used more antimicrobials than younger patients in our study, and this coincided with a higher percentage of these patients being admitted to a hospital for their infection. It is likely to be due to the fact that older people have a reduced functioning of their immune-system and that elderly get dehydrated easier(30), and are therefore more likely to be seriously affected by an infection like *Salmonella.*(31) However, of the youngest age group (<4 yrs), 35% (9 out of 26) were treated with antibiotics, which is a cause for concern. Asthma was the most common comorbidity in our study group, and these patients are more likely to suffer from upper-respiratory illnesses(32), which might be treated with antimicrobials and this might add to the antimicrobial use of patients in our study, since we did not ask specifically for which indication antimicrobials were prescribed in the six months prior to their *Salmonella* infection

In contrast to other studies, we were not able to show convincingly that infections with antimicrobial resistant *Salmonella* have a worse outcome of disease than infections with a fully susceptible antimicrobial profile. In

fact, we only found a significantly worse outcome for vomiting and for feeling nauseated when comparing R versus S, and we did not find an increasing trend with increasing antimicrobial resistance (R vs. MR). It is therefore possible that even the higher risk of vomiting and nausea was a spurious observation.

Other investigators have shown that being infected with an antimicrobial resistant *Salmonella* leads to a worse outcome of disease, including higher risk of bacteraemia(33), higher mortality(34;35), excess hospitalisation(36;37) and more infections due to therapy failure.(38) Most likely this difference is due to our study set-up which focused on softer outcomes than the abovementioned ones. The studies of Helms, Lee, Giamarellos-Bourboulis and Varma et. al., included hospitalised patients or had hospitalisation as a major outcome of the study, whereas we included outcomes as days of diarrhoea, vomiting, weight loss, and other less serious events. Furthermore, we included a lower number of patients compared with the higher-powered registry based studies and this represents another limitation In the present study, interviews were held relatively quick after onset of disease, some of our patients were still ill during the interview, and we did not look at long-term outcome of disease.

We considered adding a group of patients that were resistant to quinolones only, similar to an earlier study performed by Hald et al.(9) Since quinolones are the standard drugs of choice for treating salmonellosis, it would be of interest to look at these patients specifically. Due to the low resistance rates to this drug found in Denmark, (<1% for cases in Denmark(25) and only two in the present study) we were unable to create a quinolone-resistant group.

A draw-back of the study design was the possible selection of patients that participated in this study. The interviewers called the patients in the evening-hours (4-9pm), so we ran little risk of missing people due to their working-hours. The interviewers managed to interview 66% of the 228 reported cases (or 70% after omitting 15 patients who were infected abroad and thus not eligible for the interview). This is, in our opinion, a satisfactory participation rate, but nonetheless it is likely that there is a bias towards excluding the most severely ill. It is noteworthy that 13 patients were too sick to participate and that four patients died before they could be interviewed, Figure 1. Second, our interview was reasonably long (approximately 20-30 minutes), and not everyone was willing to commit to such a long interview.

In conclusion, we were not able to demonstrate major negative outcomes of *Salmonella* Typhimurium infection when comparing patients infected by strains classified according to three different levels of drug-resistance. This negative finding is likely to be a limitation by design of the study rather than a true absence of a detrimental effect. We recommend that high-powered studies taking advantage of electronic health records or other databases as more appropriate to address research questions regarding the public health effect of resistance than work-intensive interview studies such as the present one. To our surprise, we found that half of the patients interviewed had received an antimicrobial treatment, which is a concern in light of the need to advocate prudent use of antimicrobials. Finally, a higher risk of weight loss, hospital admission or therapy among patients with a history of antimicrobial treatment independent of the current episode adds to studies that have demonstrated competitive and selective effects of antibiotic treatment.

Acknowledgements

Financial support: This work was supported by the Danish Food Industry Agency (grant number: 3304-FVFP-07-721-01).

Other support: the authors like to thank L. Müller for her help with setting up the interview.

Conflict of interest

The authors have no conflicts of interest to declare.

Figure 1: Flow diagram of patient enrolment.

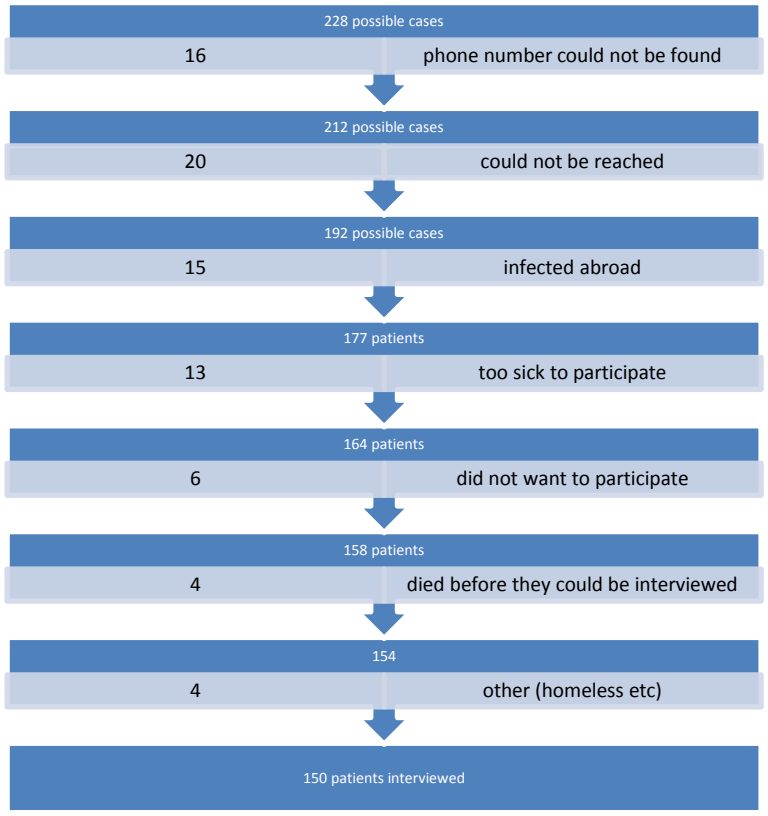

Table 1 Odds for severity of disease per resistance profile (three levels), 150 *S.* Typhimurium patients, January-June 2010, Denmark

	Three levels of resistance						All patients	
	Pansusceptible (S)*		Resistant (R)*		Multidrug-resistant (MR)*			
	N	OR (95%CI)	N	OR (95%CI)	N	OR (95%CI)	N	(%)
Total number of patients	49		48		53		150	100
Median age (interquartile range)	26	(3-51)	49	(23-65)	29	(11-55)	36	(10-57)
Age category[a]		1 (reference)		3.04 (1.40-6.56)		2.23 (1.06-1.68)		
Infants (<4y)	15	3.44 (1.36-8.70)	6	0.52 (0.19-1.46)	5	0.44 (0.15-1.30)	26	17.3
Children (4-17y)	8	1.35 (0.50-3.64)	4	0.37 (0.11-1.19)	11	1.69 (0.66-4.36)	23	15.3
Adults (18-64y)	21	1 (reference)	27	1 (reference)	26	1 (reference)	74	49.3
Seniors (=> 65y)	5	0.66 (0.22-2.01)	10	1.24 (0.49-3.18)	9	1.11 (0.43-2.88)	24	16.0
Gender (female)	25	1 (reference)	23	1.13 (0.51-2.51)	26	1.08 (0.50-2.53)	74	49.3
Any underlying comorbidity	12	1 (reference)	21	2.40 (1.00-5.70)	27	3.20 (1.38-7.45)	60	40.0
Median days of diarrhoea (interquartile range)	14	(7-15)	10	(7-14)	14	(8-20)	13	(7-15)
Severe diarrhoea (>7 days)	33	1 (reference)	27	0.62 (0.27 -1.43)	43	2.09 (0.84 - 5.62)	103	68.7
Weight loss (>5kg)	8	1 (reference)	14	0.49 (0.18-1.33)	13	0.58 (0.21-1.58)	35	23.3
Hospitalised due to infection	11	1 (reference)	20	2.47 (1.02 - 5.97)	18	1.68 (0.70 - 4.04)	49	32.7
Received medication to treat infection	22	1 (reference)	29	0.53 (0.24-1.20)	25	0.91 (0.42-1.99)	76	50.7
History of antimicrobial use[b]	20	1 (reference)	23	1.33 (0.60-2.98)	25	1.30 (0.59-2.83)	68	45.3
Vomiting (>7 days)	12	1 (reference)	22	2.61 (1.10-6.19)	20	1.87 (0.79-4.40)	54	36.0
Nausea >7 days)	18	1 (reference)	30	2.87 (1.26-6.54)	27	1.79 (0.81-3.95)	75	50.0
Abdominal pain (>7 days)	39	1 (reference)	41	1.50 (0.52-4.33)	42	0.98 (0.38-2.56)	122	81.3
Fever (>7 days)	36	1 (reference)	35	0.97 (0.40-2.39)	39	1.01 (0.42-2.43)	110	73.3
Bloody faeces (>7 days)	19	1 (reference)	20	1.1 (0.50-2.54)	20	0.96 (0.43-2.13)	59	39.3
Pain in joints	13	1 (reference)	11	0.82 (0.33-2.08)	15	1.09 (0.46-2.61)	39	26.0

* S: pansusceptible *Salmonella,* R: *Salmonella* resistant to 1-3 antimicrobials, MR: *Salmonella* strains resistant to =>4 antimicrobials

a: Age category was analysed separately within the different susceptibility profiles with adults as the reference value

b: Antimicrobials were not prescribed for treatment of current infection, history up to 6 months.

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Table 2 Relation between comorbidity and resistance profile, crude outcomes and adjusted for age category

Resistance profile	Comorbidity present	N (%)	Crude OR (95%CI)	adjusted OR* (95%CI)
S	Yes	12 (24)	1 (Reference)	1 (Reference)
	No	37 (76)		
R	Yes	21 (44)	2.40 (1.01 - 5.70)	1.73 (0.68 - 4.35)
	No	27 (56)		
MR	Yes	27 (51)	3.20 (1.38 - 7.45)	2.62 (1.07 - 6.37)
	No	26 (49)		
< 4 years	Yes	4 (15)	1 (Reference)	1 (Reference)
	No	22 (84)		
4-17 years	Yes	6 (26)	1.94 (0.47 - 7.99)	1.52 (0.36 - 6.48)
	No	17 (74)		
18-64 years	Yes	33 (43)	4.12 (1.30 - 13.12)	3.37 (1.03 - 11.01)
	No	44 (57)		
=> 65 years	Yes	7 (29)	13.36 (3.35 - 53.18)	10.89 (2.66 - 44.61)
	No	17 (71)		
Total				

*Comorbidity by resistance profile adjusted for age category

**: Comorbidity is a yes/no variable consisting of having any of the following underlying diseases: asthma/bronchitis, heart and circulation disease, intestinal illness, recurrent diarrhoea, liver disease, diabetes, connective tissue disease, kidney problems, cancer, chronic infection, other disease.

Table 3 Crude and adjusted odds ratios for people who received antimicrobials in the past 6 months prior to *Salmonella* infection

		History of antimicrobial use, unrelated to the current salmonellosis		Odds Ratio for severe outcome (95%CI)	
Consequence of infection:		Yes	No	Crude	Adjusted*
Prescribed antimicrobials	yes	52	24	7.85 (3.77 - 16.78)	7.56 (3.42 - 16.70)
	no	16	58	1 (reference)	1 (reference)
Hospitalised	yes	28	21	2.03 (1.02 - 4.06)	2.00 (0.97 - 4.11)
	no	40	61	1 (reference)	1 (reference)
Weight loss (>5kg)	yes	22	39	2.43 (1.09 - 5.48)	2.01 (0.84 - 4.82)
	no	56	13	1 (reference)	1 (reference)

* We adjusted for age category, income, level of schooling, self-reported stress, and smoking

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Appendix: Translation of the interview

Interviewer:

Interview date:

Patients name:

Age:

Region:

Telephone number:

1.1) Who is answering the questionnaire?
__ Patient ___mother/father ___ other
1.2) What was the date of first onset of your symptoms of disease?
1.3) Have you had another microbiologically proven *Salmonella* infection 6 months before this current *Salmonella* infection?
1.4) Do you have a suspicion of which food-item made you ill?
If yes, what was it?
And when did you eat it?

Period after infection

2.1) How many days passed between the first symptoms and the stool-sampling?

2.2)How many days were you ill?

2.3) Were you hospitalised due to your salmonellosis?

2.4) Did you receive antibiotics to treat your salmonellosis??

If yes, do you know the name of the antibiotic?

2.5) Did you take any other drugs for your salmonellosis?

2.6) Did you have any of the following symptoms?

Symptoms	yes	no	Not sure	Number of days
Diarrhoea				
Blood in stool				
Nausea/vomiting				
Pain in stomach				
Fever				

other				

If other, which?

2.7) Did you lose weight due to your salmonellosis?

If yes, how much? __>5kg __2-5kg ___0-2kg

2.8) Did you have pain in your joints due to your salmonellosis?

2.9) Do you have any of the following symptoms?

Disease	Yes	No	Not sure
Asthma/bronchitis			
Heart- circulation disease			
Stomach disease (f. ex. gastritis, ulcer)			
Intestinal disease (IBS, Chrohn's, collitis ulcerosa)			
Returning diarrhoea of unknown cause			
Liver disease			
Diabetes			
Connective tissue disease (f.ex. arthritis, Sjögren's syndrome)			
Kidney disease			
Cancer			
Chronic infection (f.ex. HIV, TB, hepatitis)			
Other disease			

2.10) Did you take any of the following drugs in the past 6 months?

Drugs	Yes	No	Not sure
Painkillers			
Antibiotics			
Diarrhoea-stoppers			
Painkillers			
Antacids			

Period before infection

3.1) Did you undertake any foreign travel up to 14 days before onset of disease?

If yes: Where did you travel to?

What were the travel-dates?

Socio-economic status and other

4.1) Are you vegetarian?

4.2) How many other people do you share your household with?

How old are your housemates?

4.3) What is your highest level of education?

4.4) What is your households yearly income?

4.5) Do you smoke?

4.6) Did you experience high levels of stress in the last 6 months?

If yes, did stay home because of these high stress levels?