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Food safety and zoonotic enteric pathogens: sources, risk factors and transmission routes of human salmonellosis and campylobacteriosis

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General introduction

General introduction

1. EU FOOD SAFETY GENERAL LEGAL FRAMEWORK

Food safety can be defined as the condition which ensures that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use [1]. Following a series of headline-hitting food safety crises in the late 1990s, it became apparent that national regulations on their own were no longer able to provide sufficient consumer protection in a globalized world. At the European level, legislation has therefore been enacted, transposed into national provisions and supplemented as needed. The European Commission's White Paper on food safety in 2000 was the driver towards a new structure for food safety in the European Union (EU). It presented a new concept for Europe's consumer protection based on the safety of all relevant stages of the food supply chain, i.e. the somewhat commonplace used concept "from farm to fork".

Regulation (EC) No. 178/2002 and Regulation (EC) No. 882/2004 are the legal foundations for food legislation in the EU and apply directly in all EU Member States (MSs) without having to enact national laws, providing European consumers with an uniform level of food safety. Specifically, Regulation (EC) No. 178/2002 lays down the general principles and requirements of food law in the EU as based on the farm-to-fork concept. Moreover, it establishes the remits of the European Food Safety Authority (EFSA) and has created the Rapid Alert System for Food and Feed (RASFF) network. Regulation (EC) No. 882/2004 lays down the general principles of official controls to be performed to ensure compliance with food and feed law in the EU [1].

2. FOOD-BORNE DISEASES

2.1. Public health impact

Consumers are threatened by more than 200 known pathogenic agents transmissible through food [2], including, among others, a wide range of viruses, bacteria, parasites and prions with known zoonotic potential, that is, transmissible between animals and humans. Most of these food-borne zoonotic pathogens are commonly found in the intestines of healthy food-producing animals and typically present in humans with acute gastroenteritis [2]. Gastrointestinal symptoms due to food-borne disease are generally mild to moderate in severity and self-limiting in persistence, lasting only a few days. This lends food-borne diseases to be sometimes regarded as comedy diseases, not pleasant to have or to talk about, but something more than a mere inconvenience [3]. Yet trivializing food-borne diseases ignores their magnitude and potential life-threatening complications or long-term sequelae. Annual estimates of food-borne diseases vary from 76 million cases in the United States of America (USA) [4] to 5.4 million in Australia [5], 1.3 million in England and Wales [6] and 680 thousand in The Netherlands [7]. Complications of food-borne diseases may involve severe dehydration, gastrointestinal perforation, septicaemia, renal failure, hepatitis and neurological syndromes [2,4,8]. In addition, several food-borne diseases have been associated with chronic sequelae such as irritable bowel syndrome (IBS) [8–11], inflammatory bowel disease (IBD) [11,12], reactive arthritis [8,11,13] and Guillain-Barré syndrome [8,14].

Although the global burden of food-borne diseases is currently unknown, the World Health Organization (WHO) has estimated that diarrhoeal diseases alone (a considerable proportion of which is food-borne) account for ~73 million of disability-adjusted life years (DALYs), a measure of disease burden expressed as the number of years lost by the global population due to illness, disability or early death imputable to the disease in question [15]. Moreover, economic losses due to the direct and indirect costs of food-borne diseases, including medical care, patients' absence from work or school, disposal of contaminated food and food sales

drop due to consumer reaction to food safety crises, may be considerable as well. For instance, it has been estimated that, collectively, the human disease costs for seven common food-borne pathogens (*Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli* O157:H7. *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus* and *Toxoplasma gondii*) in the USA account for 6.5–34.9 billion of 1995 USA dollars annually [16]. These are some of the reasons as to why, over the last decades, food-borne diseases have significantly moved up the political agenda of the industrialized world and generated, on occasions, substantial scientific and media attention [17].

Food-borne diseases have a complex, dynamic nature. Not only because of the many pathogens and related clinical outcomes, but also because of the wide range of foods serving as sources of human infection and animals acting as reservoirs for food-borne pathogens, as well as the numerous factors that may affect contamination, growth, persistence, and inactivation of pathogens themselves throughout the farm-to-fork continuum [18]. Despite considerable research efforts leading to a generation of new or improved methods for detecting and characterizing food-borne pathogens, supporting public health risk assessments and policy development as well as implementing effective intervention strategies such as vaccination for food-producing animals or post-harvest treatments [17], safe food can never be taken for granted. It is simply impossible to test every single food item for every imaginable pathogen, not to mention that this would make our food prohibitively expensive. Moreover, the epidemiology of food-borne pathogens changes continuously: known pathogens may be transmitted by hitherto unknown vehicles while new pathogens continue to emerge. Population growth and demographic shift towards an ageing and more susceptible population, globalization of the food supply, changing eating habits, farming practices and food technologies, and even climate change, have been proposed as factors driving the ever-changing epidemiology of food-borne pathogens [17,19,20]. It is therefore extremely important to strengthen research and improve public health surveillance of food-borne pathogens in order to monitor what is going on in the population and to empower decision makers to guide and manage more effectively by providing timely, useful evidence.

2.2. Surveillance in humans

Public health surveillance is defined as the ongoing systematic collection, analysis, and interpretation of data, with their timely dissemination to those responsible for preventing and controlling the disease in question [21]. Recent developments in the field of food-borne disease epidemiology are also the result of improvements in surveillance systems. The most widely used measure of the magnitude of food-borne diseases in a population is the estimation of the incidence of cases infected with specific pathogens. Most frequently used as a basis for such estimates is the incidence of laboratory-confirmed cases of specific pathogens usually captured by passive surveillance of notifiable diseases. This type of surveillance typically collects aetiological information on food-borne pathogens affecting only a small proportion of patients with (severe) gastrointestinal symptoms that seek for medical care, with subsequent laboratory testing for selected ranges of gastrointestinal pathogens. However, it has been shown that the laboratory tests requested by physicians do not always comply with existing knowledge of the aetiology of acute gastroenteritis [22]. Furthermore, laboratory capacity may not be standardized over the different diagnosing laboratories, as may be also the case for the reporting of cases to the surveillance systems [18]. As a consequence, the magnitude of food-borne diseases, as observed by passive surveillance, represents only the tip of the iceberg of the actual magnitude of such diseases in the population. For instance, in the EU, over 320,000 human cases of zoonotic food-borne diseases are reported each year by the EU MSs to the European Surveillance System (TESSy), but the real number is likely to be ~100 times higher [7].

Approaches to estimate the degree of under-ascertainment, or under-reporting, of pathogen-specific gastroenteritis cases in the population have been developed, allowing for the reconstruction of the so-called surveillance pyramid (Figure 1) and the estimation of the real community incidence of the major food-borne diseases in the EU [7,23,24]. Other surveillance systems may, instead, primarily target syndromes related to food-borne diseases (i.e. acute gastroenteritis) or over-the-counter medication sales (i.e. antidiarrhoeals

and antiemetics), two useful systems for early warning of community outbreaks, e.g. [25–27], especially in emergency situations, such as natural disasters [28] or unusual mass gatherings [29]. Finally, active surveillance of selected pathogens of greatest interest in given populations may be implemented to fill specific gaps in knowledge, e.g. severe rotavirus gastroenteritis in children [30]. Indeed, quantifying the impact of food-borne diseases on a given population is complicated, perhaps increasingly so, by a number of factors such as different susceptibility to (symptomatic) infection of existing (sub)populations (i.e. children, elderly, immunocompromised people, pregnant women, etc.) or different genetic traits (e.g. set of virulence and antimicrobial resistance genes) within the same pathogen species or types, which may significantly affect the severity of clinical symptoms and the effectiveness of medical treatment, not to mention the changes in consumers' behaviours regarding exposure to pathogens [18]. In such situations, extrapolations from a surveillance system to the whole population may therefore require further adjustments and special consideration.

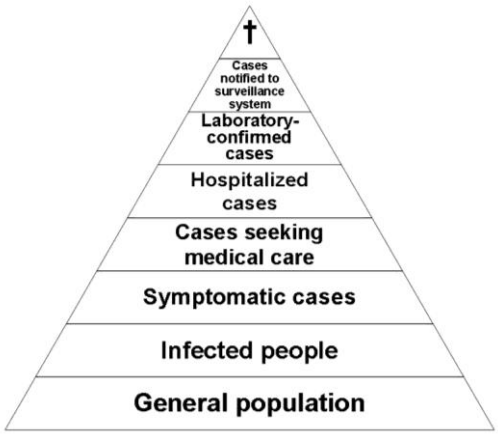

Figure 1. Surveillance pyramid of food-borne diseases.

2.2. Human illness source attribution

Source attribution is defined as the estimation of the relative contributions (partitioning) of different sources of human infection to the human disease burden [31]. Source attribution is a growing area of research that incorporates an increasing number of methodological approaches and data sources. A detailed discussion of source attribution applications, including their advantages and limitations, may be found elsewhere [31,32]. The term *source* is often used as a collective term to cover any point along the transmission chain, such as the animal reservoir or amplifying host (e.g. chicken, cattle, etc.), the vehicle (e.g. food, water, direct contact, etc.) and even specific food items (e.g. meat, milk, eggs, etc.). However, for the purposes of source attribution, a specific terminology is generally used.

- **Reservoirs or amplifying hosts**: these are animals or non-animal sources upon which the pathogen depends for its survival that can be grouped or subdivided into epidemiologically meaningful categories depending on the question being addressed. For instance, cattle, sheep and goat may be grouped together as ruminants if it is not relevant or possible to determine their independent contributions. Alternatively, poultry may be subdivided according to the supplier. Source attribution at the reservoir level provides estimates of the relative contributions of the amplifying hosts to the human disease burden for the purposes of targeting interventions at the top of the transmission route. In such attribution models, it may also be appropriate to use non-animal sources, such as the environment (e.g. water samples) to capture also the contribution from unmeasured hosts or group of hosts, such as wildlife.
- **Routes or pathways** (of transmission): these may be considered the primary ways by which pathogens shed by the reservoirs reach and infect humans. Again these can either be grouped or subdivided according to the question being addressed. Meaningful categories for informing policy are food, environment, water (which may be considered part of the environmental pathway) and direct contact. A number of approaches have been used to estimate the contribution of different pathways. Top-down approaches, which subdivide the contribution of amplifying hosts into food and environmental pathways; or bottom-up approaches, which combine the contributions from different exposures and risk factors.
- **Exposures:** primary pathways can be subdivided into a number of secondary exposures. For instance, the food pathways can be divided into meat and milk, while environmental contamination of surface water may affect drinking-water and recreational water.
- **Risk factors:** these are characteristics, conditions or behaviours that increase the probability of disease. For instance, in case-

control studies, variables are measured that describe specific determinants of risk (such as the consumption of a specific food item). The magnitude of such risk associated with these factors is estimated and the statistical significance of association is tested. These are represented as a further subdivision of pathways and exposures. For example, cattle (reservoir) may contaminate the food chain (pathway) resulting in hazard in the milk supply (exposure), which manifests itself as an increased risk associated with the consumption of unpasteurized milk (risk factor).

Attributing human infections to specific sources is crucial to inform policies for food-borne disease prevention and control. Specifically, source attribution is used to prioritize and measure the impact of targeted interventions for food-borne diseases, as well as to identify the most promising points of the transmission chain where such interventions should be targeted [31,32]. A number of approaches (reviewed by Pires *et al*. [31]) can be used for source attribution, including microbial subtyping, outbreak summary data, epidemiological studies, comparative exposure assessment, and structured expert opinion. These approaches can be broadly divided into epidemiological and microbiological approaches, and their utility varies according to data availability and research question being addressed [32].

For most source attribution studies on human salmonellosis and campylobacteriosis (the two food-borne diseases on which this thesis is focused), the microbial subtyping approach is the method of choice. This approach compares the distributions of microbial subtypes in human cases with those isolated from a range of animal, food and environmental sources to estimate the contribution of each source to the human disease burden. Data generated by either phenotypic or genotypic typing methods are of considerable value for understanding the epidemiology of food-borne diseases by refining knowledge on the relative contributions of reservoirs, pathways, exposures and risk factors in source attribution models. In particular, they provide a means of monitoring changes in reservoir attribution and epidemiology over space and time, which is of particular value for assessing the impact of different public health interventions. However, the disadvantages include the costs of sampling, isolation and genotyping of isolates which, if not already integrated in existing surveillance programmes, may be prohibitive in most cases.

A number of modelling tools are nowadays available for source attribution using the microbial subtyping approach. These models will be presented in detail throughout this thesis and are briefly introduced here as follows:

- **Proportional Similarity Index (PSI) or Czekanowski index:** this is an objective and simple estimate of the area of intersection between two frequency distributions of microbial subtypes [33,34]; thus, it can be used to assess the (dis)similarity of such frequency distributions between reservoirs and human cases. The PSI ranges from 0 (no common subtypes) to 1 (identical distribution). Confidence intervals can be approximated by bootstrapping.
- **Dutch model:** this method compares the number of reported human cases caused by a particular subtype with the relative occurrence of that subtype in each reservoir [35]. This model assumes an equal impact of the different subtypes and sources on human cases. It is easy to apply and the method of Garret *et al*. [36] can be extended to provide bootstrap confidence intervals.
- **Hald model and modified Hald model:** the Hald model is a Bayesian risk assessment model, originally developed to quantify the contribution of different food sources to human salmonellosis cases in Denmark [37]. Afterwards, this model has been modified and adapted to data of different origin and diseases other than salmonellosis, such as campylobacteriosis [34]. The original model compares the number of human cases caused by different types with their prevalence in different food sources, weighted by the amount of food consumed, accounting for differences in subtypes and sources to cause diseases in humans. This is a Bayesian development of the earlier Dutch model and requires a heterogeneous distribution of some of the frequently occurring types among the sources. By using a Bayesian approach, the Hald model can explicitly include and quantify the uncertainty around each of the parameters. The modified Hald model overcomes some of the problems of the original model associated with over-parameterization and incorporates uncertainty in the prevalence matrix [34]. Other modifications of this model have been developed and successfully applied to

salmonellosis in Sweden [38], France [39] and the USA [40], and to listeriosis in England and Wales [41].

- **Asymmetric Island (AI) model:** this is a population genetics approach and is fundamentally different from the Dutch and Hald models. It is a model of gene flow derived from population genetics that reconstructs the genealogical history of the isolates, based on their allelic profiles, and estimates mutation and recombination rates, as well as the migration rates from each reservoir into the human "islad" [42]. These migration rates are then used to estimate the relative contribution from each of the reservoirs. This technique has one major advantage over the other methods as it can assign human cases infected with subtypes that have no identified animal or environmental reservoirs.
- **Dynamic attribution model**: this model describes how reservoir attribution changes over time, and can be used for ongoing surveillance and for assessing the impact of interventions [43,44]. The Hald model forms the basis of current dynamic attribution models, and various ways by which the classical output of the Hald models may be improved have been developed, e.g. [45].

A critical issue of source attribution modelling is the point of attribution, that is, the location along the farm-to-fork continuum that is addressed by a given attribution approach. For instance, attribution focused at the point of production would identify the food-producing animal reservoirs of on-farm microbiological contamination prior or during harvesting, whereas attribution at the point of consumption or exposure would identify foods as they are prepared and eaten. Different types of data and different analyses may point to different points of attribution, and even the same type of data may point to multiple points of attribution. Because pathogens that cause food-borne diseases may enter the food chain at different points, the burden of disease attributed to specific sources may vary from one point to another. For example, attribution of human *Campylobacter* infections may partition more illness to the chicken reservoir than to broiler meat at the point of consumption since other foods, e.g. raw vegetables, may become cross-contaminated during food preparation. The point of attribution essentially depends on the method chosen and the data used. Figure 2 presents the major transmission routes for food-borne infections and indicates at which point in the transmission chain the different approaches can attribute human illness.

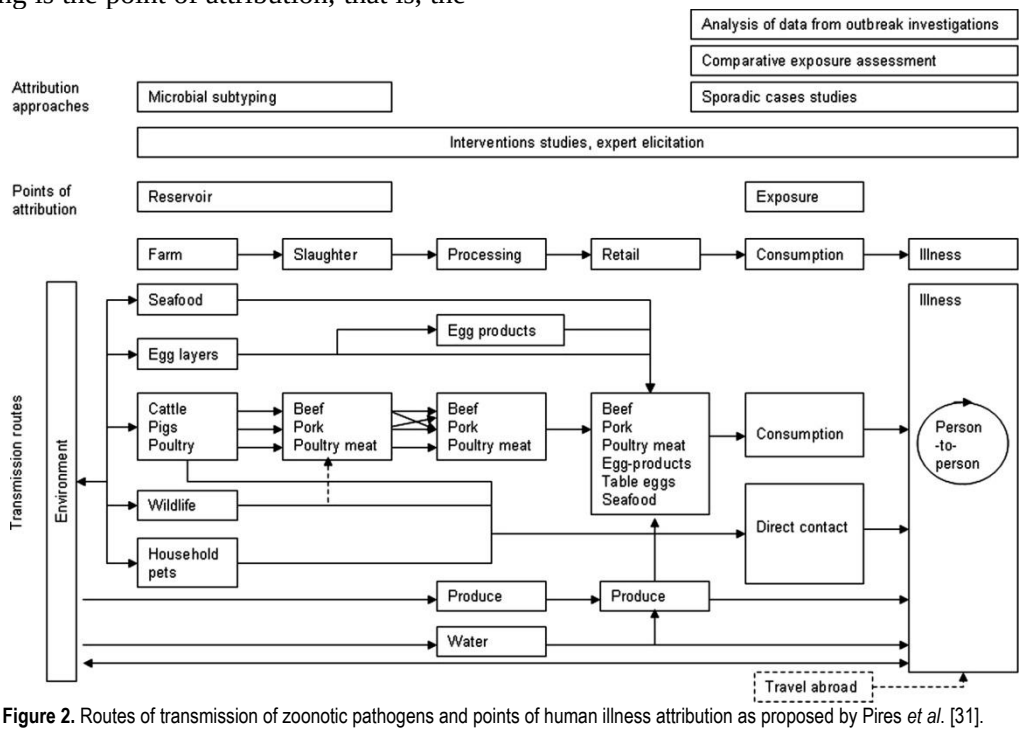

Figure 2. Routes of transmission of zoonotic pathogens and points of human illness attribution as proposed by Pires *et al.* [31].

The food system is dynamic in nature, meaning that attribution estimates rapidly become out of date. It is largely unclear how to interpret apparent trends moving forward or how to aggregate data over time. Changes in

the durable immunity of the population or of the antimicrobial resistance of pathogens can, to some extent, reasonably affect attribution estimates, as do changes in consumption patterns and changes in contamination due to regulatory changes or implementation of intervention strategies [31].

3. HUMAN SALMONELLOSIS AND CAMPYLOBACTERIOSIS

Throughout the 1990s until today, the two most reported zoonotic food-borne bacteria in the industrialized world, *Salmonella* spp. and *Campylobacter* spp., have dominated the most research and surveillance attention from government agencies and, to a large extent, the most awareness from the food industry. These pathogens contribute to the greatest burden of food-borne diseases for which aetiology is known [7,23] and provide an example of the persistence of food-borne pathogens despite considerable efforts aimed at their prevention and control in the food chain. Not surprisingly, therefore, that human salmonellosis and campylobacteriosis nowadays command the majority of public health interest.

3.1. *Salmonella*

Salmonella is a genus of Gram-negative, facultative anaerobic, rod-shaped, non-spore forming and predominantly motile bacteria (diameter 0.7–1.5 µm, length 2–5 µm) belonging to the *Enterobacteriaceae* family. *Salmonella* was first reported in 1885 by (and named after) Dr. Daniel Elmer Salmon (1850–1919), an American veterinary pathologist. The genus *Salmonella* is divided into two species, *S. enterica* and *S. bongori*, with the species *S. enterica* being further divided into six subspecies (*S. enterica* subspp. *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*).

Serotyping is used to differentiate *Salmonella* isolates beyond the subspecies level. Serotypes (or serovars) are designated based on the immunoreactivity of the O and H antigens. A considerable amount of diversity exists in these two antigens, resulting in the designation of more than 2500 known *Salmonella* serotypes and the regular recognition of new serotypes. The simplified antigenic formulae of these serotypes are listed in a document called the Kauffmann-White scheme [46], and the WHO Collaborating Centre for Reference and Research on *Salmonella*, at the Institut Pasteur in Paris, France, is in charge of updating this scheme [47]. Salmonellas most frequently transmitted through food are often referred to as non-typhoid to differentiate them from *S.* Typhi and *S.* Paratyphi, the causative agents of the Typhoid Fever, which is restricted to human-to-human transmission. Two particular non-typhoid *Salmonella* serotypes, *S.* Enteritidis and *S.* Typhimurium, have become major causes of food-borne disease in the 1980s and 1990s in the industrialized world.

S. Enteritidis and *S.* Typhimurium (and a few others) can further be divided in a number of phage types, which indicate subsets on one serotype that are susceptible to the same lytic bacteriophages [48]. Antimicrobial susceptibility testing is often used in combination with other subtyping methods. However, antimicrobial resistance is a relatively unstable characteristic as it is often carried by horizontally transferrable genetic material (transformation, conjugation and transduction). Common methods for *Salmonella* genotyping include, but are not limited to, Pulsed-Field Gel Electrophoresis (PFGE, often considered as the gold standard in epidemiological studies), Multilocus Variable Number Tandem Repeat Analysis (MLVA), Multilocus Sequence Typing (MLST) and (multiplex-) PCR-based methods. The combined use of phenotypic and genotypic typing methods, such as serotyping, phage typing, antimicrobial resistance testing, PFGE and MLVA, allows for a very detailed comparison of *Salmonella* strains. Nevertheless, in most cases, the use of serotyping only is regarded as sufficiently discriminatory, but for frequently occurring serotypes and in outbreak investigations, the use of serotyping only is often insufficiently informative.

Salmonella spp. are capable of colonizing, usually asymptomatically, the intestines of a wide range of warm and cold blooded hosts, including virtually all the major food-producing animals (e.g. poultry, cattle, pigs, etc.), pets, wildlife, reptiles and amphibians. Salmonellas are excreted from infected animals to the environment, where they can survive for extended periods, e.g. up to 60 days in faecally contaminated water or soil [49,50]. Transmission to humans occurs mainly through consumption of food of animal origin that has been faecally contaminated during slaughtering or processing, as well as

through consumption of any edible product that has been cross-contaminated during food preparation. Human *Salmonella* infection is frequently acquired because of mishandling or undercooking of food, especially poultry, eggs, seafood and raw milk. Up to 95% of human *Salmonella* infections are indeed estimated to be food-borne [51]. Nevertheless, salmonellas can also be transferred through direct or indirect contact with animals, their waste products or anything contaminated in their environments.

A recently identified trend in human *Salmonella* infections has been an increased association of outbreaks with unusual vehicles, such as fresh produce, given that manure is frequently used as a fertiliser. Studies have also suggested that some *Salmonella* spp. have now evolved to colonize vegetables [52–54] or the environment [55]. Furthermore, food handlers infected with salmonellas can transmit them if they, for instance, do not thoroughly wash their hands after toilet visit. This is the special case of the aforementioned host-adapted serovars *S.* Typhi and *S.* Paratyphi, which indeed spread from person to person, especially in countries with deficient wastewater systems.

Human salmonellosis targets predominantly the gastrointestinal tract, causing acute gastroenteritis, with diarrhoea, abdominal pain, fever and sometime vomiting. It takes a very small amount of salmonellas to sicken a person, possibly as little as 20−200 bacterial cells, and the first signs of illness can occur within 6–72 hours (incubation period), depending on the host health status, the serotype, the inoculum and the composition of contaminated food. Antibiotic treatment is not usually required as the disease is frequently self-limiting, lasting 4–7 days. However, in high risk groups (e.g. infants and young children, elderly, transplant recipients, pregnant women and people with a weakened immune system), symptoms may be so severe to require hospitalization. Development of complications, such as severe dehydration, septicaemia and extra-intestinal infections (e.g. meningitis, endocarditis or osteomyelitis), can be life-threatening. Possible documented long-term sequelae are reactive arthritis and functional gastrointestinal disorders, such as IBS and IBD [8].

Non-typhoid *Salmonella* species were estimated to cause ~93.8 million human cases of gastroenteritis globally each year, with ~155,000 deaths [56]. Approximately 6.2 million cases/year have been estimated to occur in the EU [23] and over 1.4 million in the USA [51]. Furthermore, the Sensor study in The Netherlands [57] has been used as a basis for the calculation of the burden of salmonellosis in terms of DALYs (~7 DALYs per 100,000 population/year) [7].

In 2010 in the EU, the incidence of reported laboratory-confirmed human salmonellosis cases was 21.5 cases per 100,000 population, with a statistically significant decreasing trend since 2005 (38.2 cases per 100,000 population), a possible reflection of successful *Salmonella* control programmes in poultry [58]. Indeed, most EU MSs met their *Salmonella* reduction targets for poultry in 2010, and *Salmonella* is declining in these animal populations. *S.* Enteritidis and *S.* Typhimurium are the most frequently isolated serotypes from human cases, accounting for ~45% and ~22%, respectively, of all known serotypes in humans. Notification rate is usually highest in small children (<5 years of age), with <1% of fatal cases. A peak in the number of reported human *Salmonella* infections normally occurs in August–September, with a rapid decline in winter months. This pattern is prominent for all age groups, supporting the influence of outside weather conditions (i.e. warmer temperatures) on bacterial multiplication. The proportion of cases that are acquired domestically, upon traveling and with unknown origin is ~63%, ~11% and ~26%, respectively. Nordic countries such as Finland, Sweden and Norway usually have the highest proportions of imported cases of human salmonellosis, whereas infections seem to be mainly domestically acquired in the majority of other EU countries [58].

Food-borne outbreaks of human salmonellosis are frequently reported. This is a reflection of a low infectious dose, especially when delivered in particular low-moisture foodstuffs, such as peanut butter, infant formula, chocolate, cereal products and dried milk [59], but also an ability to grow in food and in the environment, allowing amplification and long-term survival. Such diverse habitats also provide opportunities for adaptation and evolution. This is demonstrated by the changing trends in human salmonellosis observed in recent years. For instance, during the 1980s, a peak in human salmonellosis was observed throughout the developed world. This increase was mainly due to *S.* Enteritidis phage type (PT) 4, which was epidemiologically and

microbiologically linked to eggs and poultry (layer hens). These salmonellas have indeed adapted to preferentially colonize the avian reproductive tract, persist in the ovary and oviduct and survive in layer hen's eggs [60]. Intervention measures against *S.* Enteritidis PT4, including vaccination, first of breeder flocks and then of layers, has significantly reduced egg-associated infections during the late 1990s in several European countries, but from 2000 there has been further increase in human salmonellosis, this time with non-PT4 strains, such as PT1, PT14B and PT21. Outbreak data, coupled with intensive laboratory investigations, has suggested that at least some of these strains are, once again, associated with eggs; thus, as one *Salmonella* type is controlled, others appear to evolve to fill the vacant niche. It follows, therefore, that *Salmonella* spp. are remarkably adaptable and able to evolve to respond to environmental challenges.

3.2. *Campylobacter*

Campylobacter is a genus of Gram-negative, mostly sender, motile, non-spore forming, spirally curved rods (diameter 0.2–0.5 µm, length 0.5–8 µm) belonging to the *Campylobacteraceae* family. *Campylobacter* was first described in 1886 by Dr. Theodor Escherish (1857–1911), who observed this bacterium in infants died because of a disease he named "cholera infantum", as reported by Samie *et al*. [61]. However, owing to difficulties in culturing these bacteria, they have been neglected until the first isolation from human faeces in 1972 [62]. Campylobacters have been referred to as "Vibrio like organisms" until 1963 when Sebald and Veron [63] gave the actual name of *Campylobacter* to the genus based on their shape, low DNA base composition, their micro-aerophilic growth requirement, and their non-fermentive metabolism [64]. The genus *Campylobacter* contains 16 species and six subspecies. The species *C. jejuni* and *C. coli* are those most commonly isolated from human cases, accounting for ~93% of confirmed human *Campylobacter* cases characterized at the species level in the EU [58]. Both *C. jejuni* and *C. coli* are thermophilic, oxidase, catalase and nitrate positive, sensitive to nalidix acid and resistant to cephalothin [65].

A variety of *Campylobacter* typing approaches have been developed. Originally, typing methods were based on phenotypic characteristics, such as serotyping and phage typing. These methods are still in use but have proved to have a poor discriminatory power and limited value in epidemiological and source attribution studies. Molecular techniques such as *fla*-typing, ribotyping, PFGE, Amplified Fragment Length Polymorphism (AFLP) and Random Amplified Polymorphic DNA, (RAPD) are frequently used to complement phenotypic methods. MLST has been increasingly used for *Campylobacter* genotyping. MLST involves sequencing the forward and reverse strands of seven target gene fragments (Figure 3) [66]. The genes targeted code for essential metabolic functions (i.e. housekeeping genes) and therefore they are expected to be present in all isolates. These genes are under stabilizing selection, which limits the diversity available from each gene fragment. The use of seven genes provides sufficient information to allow isolates to be genealogically grouped. Indeed, by indexing the variation present in these seven housekeeping genes, MLST allows for the identification of genetic lineages in *Campylobacter* populations. A unique sequence pattern is assigned to a sequence type (ST), while closely related STs sharing the same alleles at different loci are considered as belonging to the same clonal complex (CC), the members of which possess a common ancestor [66].

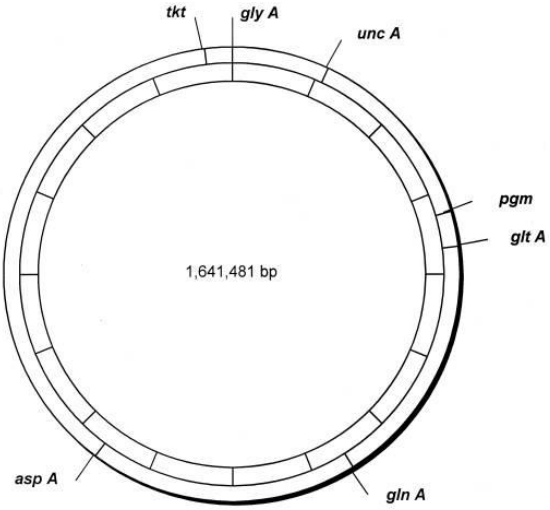

Figure 3. Chromosomal locations of the seven loci used in *C. jejuni* MLST [66].

The weakly clonal nature of campylobacters makes the use of most subtyping methods a difficult approach for tracking sources of human campylobacteriosis. In this regard, MLST has proved successful in source attribution of sporadic cases, e.g.

[34,42,67–71], as will also be shown in more detail later in this thesis.

Campylobacters are widespread in nature. They are intestinal commensal bacteria of wild and domesticated animals, especially avian species (preferential hosts), resulting in contamination of the environment, including water sources. Although *Campylobacter* spp. are mostly perceived as food-borne pathogens, there is evidence for other transmission pathways, including direct and indirect contact with infectious animals, people and environments [72–75]. Campylobacters are prevalent in food-producing animals, such as poultry, cattle, pigs and sheep, as well as in pets, including cats and dogs, in wild birds and in water sources. Animals, however, rarely succumb to symptomatic infection. The bacteria can contaminate various foodstuffs, including meat, raw milk and dairy products and less frequently fish and fishery products, mussels and fresh produce.

Case-control studies of sporadic human cases have evidenced that consumption of chicken is the most important risk factor for human campylobacteriosis [72–76]. However, as *Campylobacter* strains of chicken origin may reach humans through pathways other than food [77], the consumption and handling of chicken may account for up to 40% of human infections, while up to 80% may be attributed to the chicken reservoir as a whole [58]. Other frequently reported risk factors are consumption of unpasteurized milk [73, 75,78], eating in restaurants [78–81], contact with pets, especially puppies [76,78,80,82–84], contact with farm animals [73,76,78–80,84,85] and foreign travel [75,76,78,80–82]. Cross-contamination during food-preparation in the home has also been described as an important transmission route [86].

As *Campylobacter* is not able to multiply in foods and has a relatively long incubation period (2–5 days), contamination would less often lead to outbreaks and most cases are indeed sporadic. Large outbreaks have often been caused by consumption of unpasteurized milk and contaminated drinking water. Survival of *Campylobacter* outside the amplifying host is poor, particularly under dry, relatively warm and anaerobic conditions. However, the infective dose of these bacteria is generally low, and ~500 bacterial cells are sufficient to cause disease in humans. Patients can experience mild to severe gastrointestinal symptoms, with watery, sometimes bloody, diarrhoea, abdominal pain, fever, headache and nausea. Infections are usually self-limiting and last only a few days. Besides extra-intestinal infections, an acute *Campylobacter* infection can have serious long-term consequences, including the peripheral neuropathies Guillain-Barré syndrome and Miller-Fisher syndrome, reactive arthritis and functional gastrointestinal disorders [8,11].

Campylobacter spp. are considered to be the most common bacterial cause of human gastroenteritis in the western world. In developed countries, the organism is isolated 3–4 times more frequently from patients with gastroenteritis than *Salmonella* spp. or *E. coli*. Although scarce, data from developing countries suggest that the burden of human campylobacteriosis is considerable. Approximately 9.2 million human campylobacteriosis cases/year have been estimated to occur in the EU [23] and over 2.5 million in the USA [51]. The Sensor study in The Netherlands [57] provided a basis for estimating the burden of human campylobacteriosis in terms of DALYs (~18 DALYs per 100,000 population/year) [7]. The incidence of human campylobacteriosis was estimated to be ~9 per 1000 population/year in the United Kingdom (UK) (for 2008–2009) [87] and ~6 per 1000 population/year in The Netherlands (for 2009) [7], leading to only one out of every ~9 cases in the UK and one out of 12 in The Netherlands to be reported to national surveillance systems. In the USA, it is estimated that one out of ~30 cases is reported by FoodNet sites, and that national incidence was 1.3 million cases in 2006 [88]. These studies also indicate that one out of seven patients with campylobacteriosis in the UK, and one out of four in The Netherlands, consulted their general practitioner, a reflection of the generally severe nature of human campylobacteriosis.

Relative risks to travellers have been used to approximate the relative incidence in local residents, as recently published for *Salmonella* spp. and *Campylobacter* spp. in the EU [23]. These studies may provide a comparable estimate of the force of infection in different countries, although there are many caveats when interpreting such data. These include under-diagnosis or misdiagnosis of travel-related cases, late appearance of symptoms, absence of information on the nature and duration of travel and traveller's immunity (in particular against local endemic strains), especially as compared to the resident population.

In 2010 in the EU, the incidence of laboratory-confirmed human *Campylobacter* infections was 48.6 per 100,000 population. Children under five years of age had the highest notification rate (126.8 cases per 100,000 population). The case fatality rate was <1%. Such incidence figures lend human campylobacteriosis to be the most commonly reported gastrointestinal bacterial disease in the EU with a statistically significant increasing trend as from 2005 [58]. *Campylobacter* prevalence is usually highest in broiler meat. The proportions of cases imported from abroad, acquired domestically and with unknown origin were 6.3%, 57.2% and 36.5%, respectively. The highest number of cases is usually reported during the summer months (June–August) gradually decreasing from September to December [58].

Given the sporadic nature of human campylobacteriosis and the important role played by cross-contamination, it is very difficult to trace the sources of human *Campylobacter* infection to the original reservoirs. However, recent insights in source attribution modelling and recognition of the role of immunity in protecting against *Campylobacter* infection, together with risk assessment studies, have helped to guide risk management along the farm-to-table continuum. Some countries have indeed invested heavily in reducing human campylobacteriosis transmitted via specific food chains. Yet, from a global perspective, human campylobacteriosis remains difficult to prevent and there is an urgent need of developing alternative tools for informing public health interventions more effectively.

4. OBJECTIVES AND OUTLINE OF THE THESIS

4.1. Objectives

This thesis is focused on the epidemiology of human salmonellosis (in Italy) and human campylobacteriosis (in The Netherlands), and deals with multiple specific objectives therein. As Italy's current surveillance systems do not provide detailed epidemiological data for zoonotic enteric pathogens other than *Salmonella* spp., Dutch data on *Campylobacter* spp. were used to address the specific objectives for this pathogen. This was made possible through an ongoing collaboration between the Italian *Istituto Superiore di Sanità* (funding body of the present PhD position) and the National Institute for Public Health and the Environment (RIVM) in The Netherlands.

This thesis had the following four objectives:

1. To overview the epidemiological trends of human salmonellosis in Italy, particularly of *S. enterica* subsp. *enterica* serotypes, and to identify the most promising targets for improving the sensitivity towards pathogens causing human gastroenteritis of Italy's current surveillance systems.
2. To develop source attribution models based on the microbial subtyping approach to estimate the relative contributions of different animal and food sources to human *Salmonella* infections in Italy and to investigate possible changes in attribution estimates over different models, time periods and attribution points along the farm-to-fork continuum.
3. To develop a combined analysis of source attribution and epidemiological (case-control) data to investigate reservoir-specific risk factors for human campylobacteriosis while accounting for sampling issues and potential biases arising from source attribution in space and time.
4. To extend the combined source attribution and case-control analysis to include also factors that are not usually considered when examining likely sources of human campylobacteriosis, such as the potentially complex transmission cycles involving pets and returning travellers.

The specific objectives of this thesis do outline its structure. Indeed, this thesis is divided in two large parts according to the main pathogen in question (*Salmonella* or *Campylobacter*) and then it is further divided in seven, separate (but strictly interconnected) chapters, each of which is an article that has been published or submitted for publication in peer reviewed international journals.

4.2. Outline of part I of the thesis – Human salmonellosis (in Italy)

This part of the thesis is divided in three chapters dealing with objective No. 1 (Chapters 2 and 3) and objective No. 2 (Chapter 4).

4.2.1. Chapter 2 (or Manuscript/Article I)

In Chapter 1, trends in physician-reported gastroenteritis cases (divided in non-typhoid salmonellosis and infectious diarrhoea other than non-typhoid salmonellosis) and food-borne disease outbreaks in Italy are described using official notification data from the current national (passive) surveillance system. To identify the most promising changes to be made for improving the sensitivity towards pathogens causing gastroenteritis of Italy's current surveillance systems, a quantitative evaluation of the impact of the two recently implemented regional surveillance systems of Lombardy and Piedmont regions (in northern Italy) on the notification rates of gastroenteritis cases and food-borne disease outbreaks is also presented.

4.2.2. Chapter 3 (or Manuscript/Article II)

In Chapter 3, a detailed analysis of the trends of *S. enterica* subsp. *enterica* serovars isolated from human cases in Italy during the last 30 years is presented using data from the Italian national laboratory-based surveillance system(s) in order to identify the (re)emerging serovars and the possible causes driving the epidemiological patterns of human salmonellosis in Italy.

4.2.3. Chapter 4 (or Manuscript/Article III)

In Chapter 4, a modified version of the Dutch model and the modified Hald model for source attribution were adapted to Italian *Salmonella* data to estimate the proportions of domestic, sporadic human *Salmonella* infections in Italy attributable to four putative sources of infection (*Gallus gallus*, turkeys, pigs and ruminants) from 2002 to 2010, both at farm and food levels. A comparison of attribution estimates over different models, time periods and points of attribution was also performed.

4.3. Outline of part II of the thesis – Human campylobacteriosis (in The Netherlands)

This part of the thesis is divided in four chapters dealing with objective No. 3 (Chapters 5 and 6) and objective No. 4 (Chapters 7 and 8).

4.3.1. Chapter 5 (or Manuscript/Article IV)

In Chapter 5, several analyses based on MLST data from human and animal *C. jejuni* and *C. coli* isolates collected over 12 years in The Netherlands, together with MLST data from other countries, are performed to determine the extents of geographical and temporal biases on attribution estimates, as well as the possible methods to be used for minimizing such biases, when using non-local or non-recent MLST data for source attribution in space and time of human campylobacteriosis based on the AI model. A power-analysis is also presented to provide the minimum number of source isolates needed to perform source attribution using the AI model.

4.3.2. Chapter 6 (or Manuscript/Article V)

In Chapter 6, MLST-based source attribution of human campylobacteriosis using the AI model, and a case-control study of chicken-, ruminant- and environment-specific risk factors for human campylobacteriosis in The Netherlands derived from a newly developed analysis combining source attribution and epidemiological data, are presented.

4.3.3. Chapter 7 (or Manuscript/Article VI)

In Chapter 7, a study aimed at clarifying the role of pets (dogs and cats) in *Campylobacter* zoonotic transmission is presented. MLST-typed *C. jejuni* and *C. coli* isolates from pets and their owners are compared in a one-to-one relationship and risk factors for pet-associated human campylobacteriosis are investigated using the combined source attribution and case-control analysis developed in Chapter 6.

4.3.4. Chapter 8 (or Manuscript/Article VII)

In Chapter 8, MLST profiles of *C. jejuni* and *C. coli* strains isolated from travellers returning to The Netherlands, the risk factors potentially responsible for the acquisition of such strains upon traveling, and those potentially responsible for their secondary spread to domestic populations, are investigated by performing a case-control study on risk factors for travel-related campylobacteriosis and a combined case-control and source attribution analysis to investigate risk factors for domestically acquired campylobacteriosis caused by STs of probable exotic origin.

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Chapter 2

Surveillance of acute infectious gastroenteritis (1992–2009) and food-borne disease outbreaks (1996–2009) in Italy, with a focus on the Piedmont and Lombardy regions

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Surveillance of acute infectious gastroenteritis (1992–2009) and food-borne disease outbreaks (1996–2009) in Italy, with a focus on the Piedmont and Lombardy regions

ABSTRACT

We describe trends in the occurrence of acute infectious gastroenteritis (1992 to 2009) and food-borne disease outbreaks (1996 to 2009) in Italy. In 2002, the Piedmont region implemented a surveillance system for early detection and control of food-borne disease outbreaks; in 2004, the Lombardy region implemented a system for surveillance of all notifiable human infectious diseases. Both systems are internet based. We compared the regional figures with the national mean using official notification data provided by the National Infectious Diseases Notification System (SIMI) and the National Institute of Statistics (ISTAT), in order to provide additional information about the epidemiology of these diseases in Italy. When compared with the national mean, data from the two regional systems showed a significant increase in notification rates of non-typhoid salmonellosis and infectious diarrhoea other than non-typhoid salmonellosis, but for food-borne disease outbreaks, the increase was not statistically significant. Although the two regional systems have different objectives and structures, they showed improved sensitivity regarding notification of cases of acute infectious gastroenteritis and, to a lesser extent, food-borne disease outbreaks, and thus provide a more complete picture of the epidemiology of these diseases in Italy.

1. INTRODUCTION

Acute gastroenteritis of infectious aetiology is a public health problem worldwide [1]. Although cases in industrialised countries are usually characterised by low mortality, the economic impact on health services (direct costs) and on the general public (indirect costs) can be considerable [2]. Any initiative aimed at controlling acute infectious gastroenteritis in a population should be based on the extent of the problem. However, the true incidence of the disease in the population, based on data from national surveillance systems, is usually underestimated, e.g. [3]. In Italy and other countries, this problem can be attributed to several factors: (i) most cases have mild, self-limiting symptoms, which do not motivate patients to seek medical attention; (ii) stool examination is not always recommended by the attending physician and an aetiological diagnosis is rarely made; (iii) diagnostic capabilities and protocols differ greatly among laboratories; and (iv) under-reporting, as it is known that physicians rarely report cases.

In Italy, surveillance of acute infectious gastroenteritis and food-borne disease outbreaks is part of the activities of the Italian National Surveillance System of Infectious Diseases (SIMI), which has been in place since 1990 [4]. Notification data of cases of acute infectious gastroenteritis and food-borne disease outbreaks are also shared with the National Institute of Statistics (ISTAT), which produces official statistics on economic, social and health matters in Italy. The Piedmont and Lombardy regions, in the north of the country, have implemented two different Internet-based surveillance systems since 2002 and 2004, respectively. The Piedmont system is dedicated to surveillance of food-borne diseases, with an emphasis on outbreaks (including but not limited to acute infectious gastroenteritis, as this can frequently be caused by food-borne pathogens), whereas the Lombardy system is aimed at improving the surveillance and reporting of all notifiable human infectious diseases, including acute infectious gastroenteritis and food-borne diseases. Both systems notify to the national surveillance system. As the two regions

together account for about a quarter of the Italian population (in 2009: Piedmont: 4,432,571 inhabitants; Lombardy: 9,742,676; national: 60,045,068 [5]) estimates of disease incidence from these regional surveillance systems can be considered relevant for comparisons at the national level.

At present, the national surveillance system does not collect notifications of acute infectious gastroenteritis as one syndrome; instead, laboratory-confirmed cases of diarrhoeal disease are generally notified in two categories: non-typhoid salmonellosis (hereafter referred to as salmonellosis) and infectious diarrhoea other than salmonellosis (hereafter referred to as infectious diarrhoea). These two categories therefore include diarrhoeal diseases caused by all identified enteric pathogens. For the purposes of this article, the official notifications of salmonellosis and infectious diarrhoea were used as proxies for acute infectious gastroenteritis, but we analysed the data separately due to the large difference in the number of cases in the two categories.

Cases of salmonellosis and infectious diarrhoea are notified to the national surveillance system according to its criteria, which, for these diseases, are based on laboratory results [4]. Food-borne disease outbreaks are generally notified to the system as the occurrence of the same disease in two or more people belonging to the same community (family, school, etc.) or exposed to a common source of infection.

The aim of our analysis was to describe the epidemiology of acute infectious gastroenteritis and food-borne disease outbreaks in Italy using official notification data collected in 1992–2009 and 1996–2009, respectively. We have also taken into account the contribution of the notification data from Piedmont and Lombardy and speculated on the impact that the notifications from the two regions could have at the national level. Our findings may help decision-makers in developing novel approaches aimed at improving the surveillance of acute infectious gastroenteritis and food-borne disease outbreaks in the general population.

2. METHODS

2.1. Data collection

Notification data were obtained from the SIMI online databases from 1996 to 2009 (for salmonellosis, infectious diarrhoea and food-borne disease outbreaks) [6] and the ISTAT from 1992 to 1995 (for salmonellosis and infectious diarrhoea) [7]. Data are available on request.

The SIMI started publishing data in 1996, while data of the previous four years were made available by the ISTAT only. There were no available data on food-borne disease outbreaks before 1996. Data on salmonellosis and infectious diarrhoea were collected per year, region, age group (0–14 years, 15–24 years, 25–64 years, 65 years and older) and sex, while those on food-borne disease outbreaks were only available per year and region. Population data per year, region, age group and sex were also collected from the ISTAT.

In order to obtain information on the two regional surveillance systems, we developed a questionnaire according to guidelines provided by the United States Centers for Disease Control and Prevention [8]. The questionnaire is available on request. It was completed by the heads of the two systems.

2.2. Data analysis

Annual notification rates (annual number of notified episodes per 100,000 inhabitants) of salmonellosis and infectious diarrhoea (from 1992 to 2009) were calculated per region, age group and sex, while those of food-borne disease outbreaks (from 1996 to 2009) were calculated per region only. Age- and sex-standardised annual notification rates of salmonellosis and infectious diarrhoea were then calculated per region using 2001 population data. Rates were calculated for the Piedmont and Lombardy regions and for the country as a whole (calculated as the mean of the 20 Italian regions).

Temporal trends in annual notification rates of salmonellosis, infectious diarrhoea and outbreaks of food-borne diseases were assessed using the Cuzick test [9]. Annual rates of salmonellosis and infectious diarrhoea were compared between the sexes using the Mann-Whitney test and among age groups using the Kruskal-Wallis test. Post hoc paired comparisons after the Kruskal-Wallis test were tested using the Mann-Whitney test on each pair of age group and p-value adjustment according to Bonferroni's method [10].

To evaluate any difference in notification rates in Piedmont and Lombardy, compared with the national mean, the standardised annual notification rates of salmonellosis, infectious diarrhoea and food-borne disease outbreaks in both regions were centred on (i.e. subtracted from) the corresponding national mean and then intra-regionally compared between the periods before (Piedmont: 1992 or 1996 to 2001; Lombardy: 1992 or 1996 to 2003) and after the implementation of their respective systems (Piedmont: 2002–2009; Lombardy: 2004–2009), using the Mann-Whitney test.

Statistical analysis was performed with STATA 10.1 and Excel. Statistical significance was set at a p value of 0.05.

2.3. Regional surveillance systems

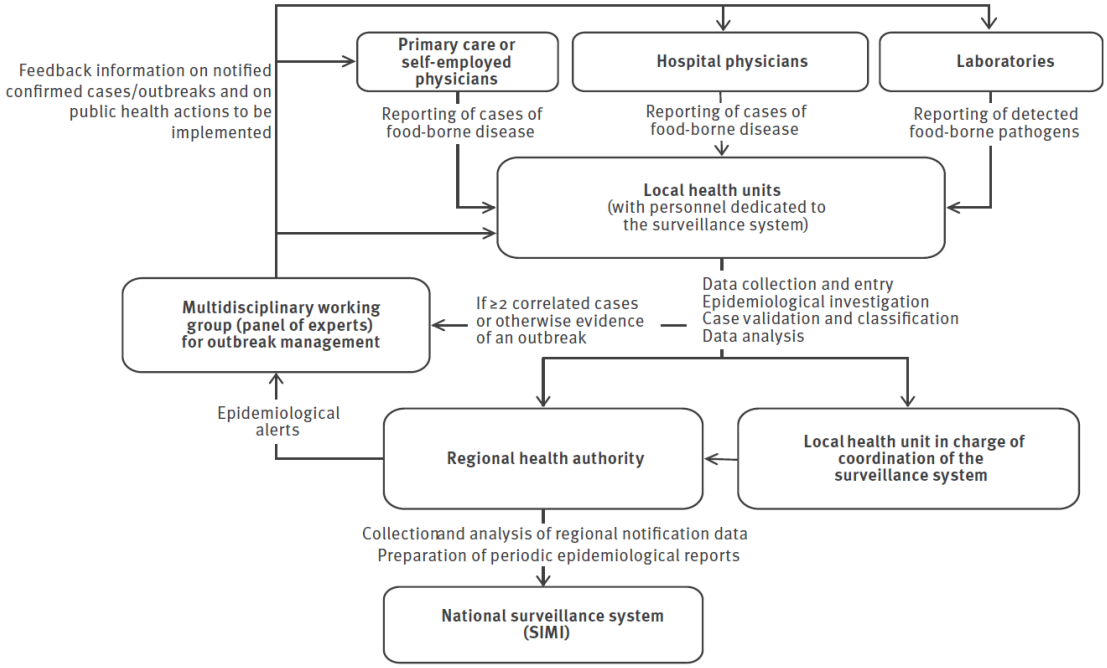
All regions other than Lombardy notify cases according to the SIMI criteria [4]. Cases notified to SIMI are not divided into possible, probable or confirmed cases, as in the European Union (EU) case definition [11]. The cases notified to the SIMI are later reported to the EU by the Ministry of Health through the European Surveillance System (TESSy). In contrast, Lombardy, uses the EU case definition, but the cases are then reported to the national surveillance system according to SIMI criteria.

2.3.1. Piedmont

The surveillance system of Piedmont is structurally independent of the SIMI. It collects data on all food-borne diseases, including episodes due to food-poisoning (e.g. those involving mushrooms, marine biotoxins and histamine) that are not notified to the SIMI. Basically, it is a passive system focused on the early detection of food-borne disease outbreaks, with the aim of improving the rapid alert and investigation of the outbreaks to prevent further cases.

Data generated from the system are also used for: (i) monitoring of spatio-temporal trends in food-borne diseases, including identification of pathogens, food items involved, related risk factors and the at-risk population; (ii) driving the development and evaluation of control programmes (for prioritising resource allocation); (iii) detecting changes in the impact of acute gastroenteritis in response to public health actions; and (iv) providing a basis for epidemiological research.

The system collects information on food-borne disease outbreaks and laboratory-confirmed individual cases of food-borne diseases, thus including salmonellosis and other diarrhoeal pathogens, which are frequently transmitted by contaminated food (Figure 1). Reporting of food-borne diseases is managed separately from other diseases. Each local health unit in the region has dedicated staff who manually enter the received data (usually by fax, email or telephone) into an Internet-based database shared by local health units and the regional health authority. Entry of all validated data is performed on a weekly basis. One person in each local health unit is in charge of validating the data, ensuring that the data are entered and coordinating a multidisciplinary panel of experts to investigate every outbreak of food-borne diseases detected by the system. In the local health unit in the city of Turin, there is a regional coordinator who is in charge of coordinating all other local health units and report to the regional health authority.



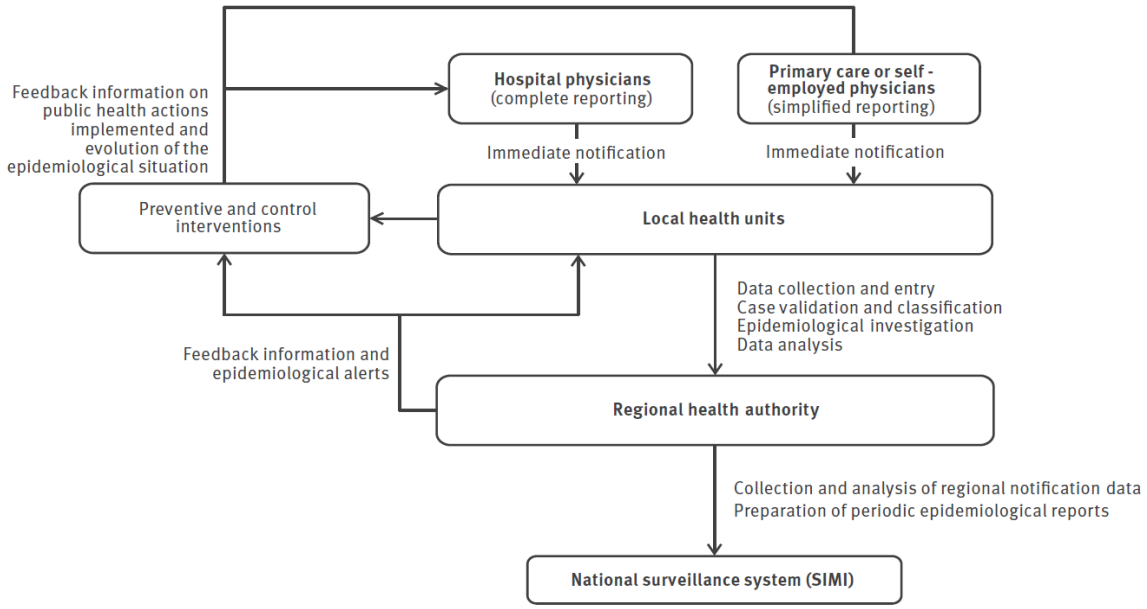
SIMI: National Infectious Diseases Notification System.

Figure 1. Surveillance system of Piedmont region, Italy.

2.3.2. Lombardy

The surveillance system of Lombardy represents an Internet-based improvement of the SIMI and it is fully integrated with it. The system has primarily been implemented to improve aetiological diagnosis and data quality for individual cases. Its main objective is to provide data for real-time analyses on spatio-temporal trends aimed at preventing secondary cases by means of prompt public health actions.

The structure of the Lombardy system (Figure 2) is basically the same as that of the SIMI, which has a pyramidal structure from the bottom (physicians) to the top (regional health authorities) and finally to the Ministry of Health, which hosts the SIMI, but compared with the SIMI, the procedure for physicians reporting to local health units was modified by: (i) reducing the information requested to a minimum (additional information requested by the SIMI for completing the notification is provided by the local health units later on); (ii) shortening the deadline for reporting (e.g. for acute infectious gastroenteritis, notification of cases should be immediate instead of within 48 hours, as required by Italian law) [4]; and (iii) defining different levels of detail required for cases detected at hospitals and for those detected by primary care or self-employed physicians. Data of the notified cases received by each local health unit are manually entered into an Internet-based database and automatically matched with the corresponding patient information stored in the regional health registry. Further epidemiological investigations are carried out when necessary. Cases are automatically validated and classified as notifiable to the SIMI or not notifiable. The database is shared among all local health units and the Lombardy regional health authority, which is in charge of the final data cleaning and analysis.



SIMI: National Infectious Diseases Notification System.

Figure 2. Surveillance system of Lombardy region, Italy.

In both systems, access to the database is restricted to authorised staff of the local health units and regional health authority. All data are managed according to Italian legislation on privacy. Both systems regularly notify to the SIMI only those cases (divided into salmonellosis and infectious diarrhoea) and food-borne outbreaks that meet the SIMI notification criteria (the set of information that must be collected in order to notify the case to the system is described in the legislation [4]).

3. RESULTS

3.1. Epidemiology of acute infectious gastroenteritis and food-borne disease outbreaks in Italy

During the period analysed (1992–2009 for salmonellosis and infectious diarrhoea and 1996–2009 for food-borne disease outbreaks), a total of 222,277 cases of salmonellosis, 46,903 cases of infectious diarrhoea and 7,937 food-borne disease outbreaks were notified in Italy. Piedmont notified 16,431 cases of salmonellosis (7.4% of the total), 4,012 cases of infectious diarrhoea (8.6%), and 570 food-borne disease outbreaks (7.2%), while Lombardy notified 43,040 cases of salmonellosis (19.4%), 14,797 cases of infectious diarrhoea (31.5%), and 1,663 food-borne disease outbreaks (21.0%). Annual notification rates of salmonellosis, infectious diarrhoea and food-borne disease outbreaks in Piedmont and Lombardy, together with the national mean, are shown in Figure 3.

3.1.1. Salmonellosis notifications

At the national level, salmonellosis notification rates significantly decreased from 47.3 per 100,000 population in 1992 to 6.7 per 100,000 population in 2009 (a decrease of 86%). Statistically significant decreasing trends were also observed in Lombardy (–58%, from 46.2 per 100,000 population in 1992 to 19.5 per 100,000 population in 2009) and Piedmont (–82%, from 47.4 per 100,000 population in 1992 to 8.6 per 100,000 population in 2009).

3.1.2. Infectious diarrhoea notifications

National notification rates of infectious diarrhoea increased significantly from 2.7 per 100,000 population in 1992 to 5.8 in 2009 (an increase of 53%). From 1992 to 2009, the annual notification rates in Piedmont increased significantly from 0.9 per 100,000 population to 7.1 per 100,000 population (+87%) and from 1.3 per 100,000 population to 30.2 per 100,000 population in Lombardy (+96%). Figure 3 shows that in both regions, notification rates of infectious diarrhoea were above the national mean from 2000 onwards.

3.1.3. Food-borne disease outbreaks notifications

The mean national notification rates of food-borne disease outbreaks significantly decreased from 1.5 per 100,000 population in 1996 to 0.4 per 100,000 population in 2009 (–73%). No statistically significant trends were detected in Lombardy (–50%, from 2.2 per 100,000 population in 1996 to 1.1 per 100,000 population in 2009), where notification rates were below the national mean from 2000 to 2006. From 1996 to 2009, there was no statistically significant trend in Piedmont, although the notification rate decreased from 2.3 per 100,000 population in 1996 to 0.2 per 100,000 population in 2009 (–91%). As shown in Figure 3, notification rates were above the national mean from 2003 to 2006, and then again in 2008, but were below the national mean in 2007 and 2009.

Significant differences in notification rates of salmonellosis and infectious diarrhoea by age group were observed in Piedmont, Lombardy and the country as a whole (Table 1). The highest notification rates were observed in children aged 0–14 years, in both regions and nationally. Apart from the 0–14year-olds, the only significant difference was observed in elderly patients (≥65 years) in Lombardy for infectious diarrhoea; in this age group the notification rates was 14.10 cases per 100,000 population in Lombardy, while in Italy and in Piedmont the rates were lower (2.84 and 4.36 per 100,000 population, respectively). No statistically significant differences were detected between male and female cases for either salmonellosis or infectious diarrhoea.

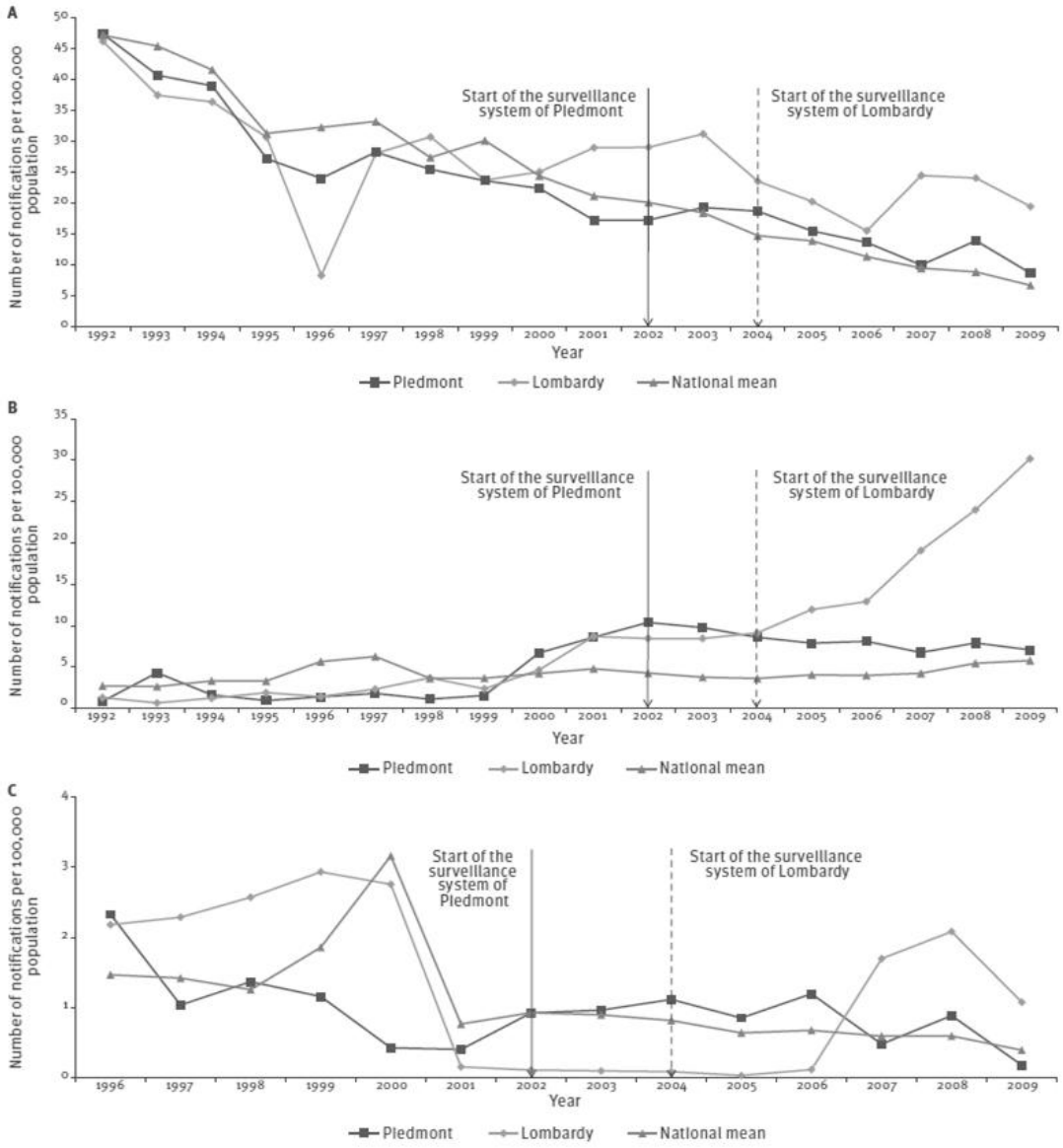


Figure 3. Trends of annual notification rates of (A) non-typhoid salmonellosis (1992–2009), (B) infectious diarrhoea other than non-typhoid salmonellosis (1992–2009) and (C) food-borne disease outbreaks (1996–2009) in Piedmont and Lombardy regions and the Italian national mean.

Table 1. Mean annual notification rates by age group and sex of non-typhoid salmonellosis and infectious diarrhoea other than non-typhoid salmonellosis, Piedmont and Lombardy regions and Italian national mean, 1992–2009.

Disease, by region or nationwide	Average annual notification rate[a]					
	Age group[b]				Sex[c]	
	0–14 years	15–24 years	25–64 years	≥65 years	Male	Female
Non-typhoid salmonellosis						
Piedmont	99.73 ± 6.09§	24.06 ± 8.00†	21.73 ± 9.04†	14.92 ± 2.12†	41.98 ± 6.50	38.24 ± 6.12
Lombardy	127.58 ± 5.9§	19.49 ± 6.13†	19.11 ± 7.35†	18.11 ± 1.71†	48.03 ± 7.10	44.12 ± 6.63
National average	98.20 ± 6.89§	32.65 ± 12.41†	24.72 ± 9.93†	17.33 ± 2.60†	44.45 ± 7.26	42.00 ± 7.21
Infectious diarrhoea other than non-typhoid salmonellosis						
Piedmont	25.80 ± 3.15§	1.36 ± 0.18†	1.49 ± 0.30†	4.36 ± 0.67†	8.83 ± 1.76	7.68 ± 1.54
Lombardy	32.43 ± 4.14§	2.85 ± 0.39†	1.97 ± 0.30†	14.10 ± 3.77‡	14.02 ± 2.62	11.66 ± 2.26
National average	19.80 ± 1.04§	1.97 ± 0.06†	1.22 ± 0.05†	2.84 ± 0.51†	7.04 ± 1.09	5.88 ± 0.89

a. Mean number of cases per 100,000 population±standard error.

b. Post hoc paired comparisons of mean annual notification rates between age groups were tested by the Mann–Whitney test. Symbols (§, † and ‡) indicate the results of the pairwise comparisons: in the same row, age groups marked with different symbols are statistically different when compared (Bonferroni-adjusted p<0.05), while the same symbol in the same row indicates no difference between the age groups.

c. No statistically significant differences between rates in male and female groups were observed (Mann-Whitney test p>0.05).

3.2. Impact of the regional surveillance systems on acute infectious gastroenteritis notification rates

Differences in notification rates from the two regions of salmonellosis, infectious diarrhoea and food-borne disease outbreaks with those of the whole of the country (national mean) before and after the implementation of the regional systems is described in Table 2. In Piedmont, after implementation of its system, there was a significant increase in notification rates of both salmonellosis (an increase of 1.6 cases per 100,000 population per year) and infectious diarrhoea (an increase of 3.9 per 100,000 population per year) compared with the national mean. In Lombardy, the increase after the implementation of its system was significant for both salmonellosis (an annual increase of 10.3 cases per 100,000 population) and infectious diarrhoea (an annual increase of 13.3 per 100,000 population). The observed increases in the notification rate of food-borne disease outbreaks after the implementation of the two regional systems (annual increases of 0.1 and 0.2 per 100,000 population in Piedmont and Lombardy, respectively) were not statistically significant.

Table 2. Differences in annual notification rates of non-typhoid salmonellosis, infectious diarrhoea other than non-typhoid salmonellosis, and food-borne disease outbreaks, Piedmont and Lombardy regions with the Italian national mean, before and after implementation of regional surveillance systems.

Disease	Differences in annual notification rate[a,b]					
	Piedmont			Lombardy		
	Before implementation (1992/1996-2001)[c]	After implementation (2002-2009)	p value	Before implementation (1992/1996-2003)[c]	After implementation (2004–2009)	p value
Non-typhoid salmonellosis	–4.05 ± 0.79	+1.58 ± 0.83	<0.01	–1.54 ± 2.79	+10.27 ± 1.87	<0.05
Infectious diarrhoea other than non-typhoid salmonellosis	–1.12 ± 0.89	+3.90 ± 0.61	<0.01	–0.25 ± 0.87	+13.34 ± 2.95	<0.01
Food-borne disease outbreaks[d]	–0.53 ± 0.49	+0.13 ± 0.08	>0.05	+0.16 ± 0.32	+0.22 ± 0.40	>0.05

a. Mean number of cases per 100,000 population ± standard error.

b. Reference value (national mean) = 0.

c. From 1992 for salmonellosis and infectious diarrhoea and from 1996 for food-borne disease outbreaks.

d. In Piedmont, includes also outbreaks due to food poisoning

4. DISCUSSION AND CONCLUSIONS

Analysis of the notifications of salmonellosis, infectious diarrhoea and food-borne disease outbreaks showed important differences between the figures provided by the regional surveillance systems of Piedmont and Lombardy and those of the national surveillance system. When we compared the regional figures with the national mean, we found significantly higher notification rates of salmonellosis and infectious diarrhoea in the two regions after the implementation of their systems. In addition to these increased rates, the absence in these two regions of the significantly decreasing trend in food-borne disease outbreaks observed at the national level can be considered a positive performance of the systems.

The better performance of the two regional systems could be related to increased motivation of those involved (e.g. physicians, epidemiologists, public health professionals and laboratory staff) to report cases of acute

infectious gastroenteritis, increased awareness of the disease and better coordination between laboratory and local health unit teams. In both regional systems, the web-based management and sharing of notification data have facilitated the reporting process and improved the completeness of the information collected. Web-based surveillance systems have become increasingly widespread and it is known that they can improve sensitivity [12–14]. Nonetheless, both Italian regional systems have major weaknesses, in particular: (i) limitations in events covered (the Piedmont system is focussed on food-borne diseases only); (ii) limitations in automatic outbreak detection (spatio-temporal clusters); and (iii) data entry is carried out far from the source. Points ii and iii, in particular, are consequences of the lack of real-time data collection and analysis and of the labour-intensive activity required by both systems. These two constraints could considerably be balanced out by full electronic reporting and management of notification data.

Concerning the epidemiology of acute infectious gastroenteritis in Italy, we identified a significantly decreasing trend of salmonellosis over the period analysed, which has also been observed in other industrialised countries, possibly resulting from improved *Salmonella* control measures in the food chain [15,16]. Although the national trend is decreasing, salmonellosis rates in Lombardy and Piedmont showed a rise from 2006 and 2007 onwards, respectively. In 2009, data provided to the European Food Safety Authority (EFSA) showed an increase in the number of *Salmonella* isolates from human cases in Italy of 22.2%, compared with those in 2008 (from 3,232 to 4,156 isolates) [16]. This increase was detected one or two years in advance by the surveillance systems of Piedmont and Lombardy (in 2008 and 2007, respectively), but not by the national surveillance system. The difference between our data and those provided to EFSA can be explained by the different sources: our data are the official notification data, while the data provided to EFSA are from Enter-net, a laboratory-based surveillance network for enteric pathogens [17].

In Lombardy, and to a lesser extent in Piedmont, the trend of salmonellosis observed during 2006 to 2009 seems related to the trend seen for food-borne disease outbreaks in the same period. Taking into account that in the EU most of the acute infectious gastroenteritis outbreaks in humans are caused by *Salmonella* [15,16], we can hypothesise that, at least in Lombardy, improved outbreak detection could have contributed to the increase of salmonellosis cases notified to the system.

The observed trends of infectious diarrhoea notification rates suggest an increasingly prominent role of pathogens other than *Salmonella* - in particular *Campylobacter jejuni* - which is the most frequent cause of acute infectious gastroenteritis in the EU [15,16]. The increasing trend of infectious diarrhoea was particularly evident in Lombardy, but was also seen in Piedmont, and could be related to the improved routine laboratory capacity for the detection and notification of pathogens other than *Salmonella*. In both regions, improvement in laboratory capacity (particularly in Lombardy) was implemented at the same time the surveillance systems were introduced. This enabled the regional diagnostic and microbiology laboratories to extend the range of assays routinely performed and pathogens searched for, and to improve the timeliness of diagnosis and their communication with the staff of the local and regional health authorities involved in the system.

Acute infectious gastroenteritis notification rates by age group confirmed the higher incidence of both salmonellosis and infectious diarrhoea in children (0–14 years), in line with what has been observed in the United States [18] and in other European countries [e.g. 19].

Concerning the trend of food-borne disease outbreaks, Lombardy showed a very low notification rate between 2001 and 2006. This is probably related to the changes in the notification procedure of such outbreaks to the SIMI (but not the notification of single cases) that Lombardy made in 2001, during the period considered for the analyses. After 2006, however, the reporting of these outbreaks was redefined, in agreement with the SIMI definitions.

In Lombardy, we observed that the implementation of the system improved notification rates of acute infectious gastroenteritis and food-borne disease outbreaks, with a reduction of the under-reporting, and consequently gave a better estimate of the impact of acute infectious gastroenteritis on the population. The Piedmont surveillance system, which is dedicated to acute infectious gastroenteritis, allows broader collection of information that is

not easy to obtain in other ways, in particular concerning food-poisoning outbreaks.

With regard to the extension of the surveillance systems of Piedmont and/or Lombardy to the other Italian regions, and even to other countries, decisions should be made on the basis of cost-benefit analyses that take into account the expected improvements in terms of efficacy of the surveillance and the resources needed to achieve them, as well as the long-term sustainability of the systems.

In conclusion, improving the surveillance of acute infectious gastroenteritis at the Italian national level requires additional efforts, which can be defined by looking at the experience at the regional level, such as that of Lombardy and Piedmont. Such efforts should be focused on the integration and harmonisation of different surveillance activities and sources of information, as well as evaluation of such activities, to obtain the best achievable impact on the burden of acute infectious gastroenteritis in the population.

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Chapter 3

Distribution of *Salmonella enterica* serovars isolated from human cases in Italy, 1980–2012

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ABSTRACT

We describe trends of *Salmonella enterica* serovars isolated from humans in Italy from January 1980 to June 2012. A total of 231,414 *Salmonella* isolates were reported. Serovars Enteritidis, Typhimurium, Infantis, Derby, 4,[5],12,:i:-, and Napoli accounted for 59% of these isolates. Temporal trends from 2000 to 2011 varied by serovar: Enteritidis and Infantis decreased significantly (–3.0% and –2.8% isolates on average per year, respectively); Typhimurium remained stable; while 4,[5],12:i:-, Derby and Napoli increased significantly (+66.4%, +8.1% and +28.2%, respectively). Since 2000, Enteritidis fell consistently below Typhimurium, which is the most reported serovar in Italy in contrast to the international situation where Enteritidis still ranks at the top despite its significant decrease. Most serovars showed a marked seasonality, increasing over the summer months and peaking in August/September. Typhimurium, 4,[5],12:i:-, and Napoli were most likely to be isolated from children, whereas Enteritidis, Derby, and Infantis from adults. We concluded that the applied control measures are not equally efficient against the considered *Salmonella* serovars and that sources of infection other than those of Enteritidis (laying hens and eggs) have become increasingly important. Further investigations on the emerging serovars and on the causes related to their emergence are needed, in order to define and implement newly tailored control measures.

1. INTRODUCTION

In the European Union (EU), *Salmonella* infection is the primary cause of confirmed food-borne outbreaks and the second most reported zoonosis, behind *Campylobacter* infection [1]. Recently, it has been estimated that approximately 6.2 million cases of human salmonellosis occur in the EU general population each year, 298,000 of which occur in Italy (~60 million population) [2].

More than 2500 serovars of *Salmonella enterica* have been described [3]. Although virtually all these serovars are capable of infecting humans, most human infections are caused by a limited number of serovars. *S.* Enteritidis and *S.* Typhimurium are amongst the serovars most frequently associated with human illness in the EU, accounting for up to 68% of confirmed human cases identified at serovar level [1]. Poultry, and particularly laying hens for table egg production, have long been identified as the primary source of human *S.* Enteritidis infection, whereas it is widely accepted that human *S.* Typhimurium infection primarily originates from pigs [4].

Salmonella serotyping is an important tool for surveillance purposes that allows for trends to be monitored over space and time. Serotyping is also a useful classification scheme to support the investigation of food-borne outbreaks and the attribution of human cases to different sources of infection and routes of transmission [4].

In Italy, the laboratory-based surveillance system for human *Salmonella* infections has changed substantially over time to follow the evolution of the surveillance activities for infectious diseases undertaken at national and international level [5]. The former system was created in 1967 and was based on the Reference Centres for Enterobacteriaceae (RCE) [5,6], which became part of the European SALM-NET (*Salmonella* Network) project later in 1992 [5]. In 1997, SALM-NET has further changed into the actual ENTER-NET (Enteric Pathogen Network) [7]. Italy's ENTER-NET is a passive, laboratory-based surveillance system for enteropathogens based on a network of more than 140 clinical microbiology diagnostic laboratories covering about 65% of the Italian territory and is complementary to the Italian National Surveillance System for Infectious Diseases (SIMI) [8,9]. Since October 2007, ENTER-

NET has been coordinated by the European Centre for Disease Prevention and Control (ECDC), European Food- and Water-borne Disease and Zoonoses Surveillance Network (FWD-Net) [10].

In Italy, ENTER-NET collects basic microbiological information (at least the serovar) on *Salmonella* isolates from human cases each year. These isolates correspond to approximately 50% of the total number of human salmonellosis cases notified to the SIMI [11]. Since 2002, the ENTER-NET laboratories are also invited to submit *S.* Enteritidis and *S.* Typhimurium isolates to the Istituto Superiore di Sanità (Italian National Institute of Health) for phage and molecular typing and antimicrobial susceptibility testing.

The aim of this study was to describe the distribution of *Salmonella* serovars isolated from humans in Italy from January 2012 to June 2012, with a focus on the six most frequently reported serovars.

2. MATERIALS AND METHODS

Data of *Salmonella* isolates from human cases were obtained from different laboratory-based surveillance systems depending on the considered time period. Data from 1980 to 1992 were obtained from published statistics of the RCE [6]. Data from 1993 to 1997 were obtained from the SALM-NET records (http://www.iss.it/salm/arch/index.php?lang=1&tipo=4&anno=2012) and those from 1998 to June 2012 from ENTER-NET (http://www.iss.it/Ente). In all of these three systems, the common case definition was "an isolate of *Salmonella enterica* with identified serovar from a human specimen".

For the purposes of this study, a minimum set of comparable information about each serotyped isolate was collected, including the patient sex, age and residence location, the laboratory that reached the microbiological diagnosis and the date of isolation thereof. This set of information was not systematically collected and made available since 2000; before 2000 only the serovar and the date of isolation were available.

A data set including *Salmonella* isolates of the whole study period (1980–June 2012) was created by merging the data obtained from the three systems (RCE, SALM-NET, and ENTER-NET). For the year 2012 only the data from 01 January to 31 June were available. This data set contained 256,022 records (i.e. isolates) with information on the serovar and date of isolation.

Another data set that included the isolates collected by ENTER-NET from 2000 to June 2012 (58,150 records) was created. This data set contained a number of duplicate entries, i.e. different isolates from a same case (because of the follow-up of patients with *Salmonella* infection after the first isolation) that were not always indicated. Therefore, duplicate entries for an isolate that matched on serovar, laboratory reaching the microbiological diagnosis, and date of birth of the patient within the same or the consecutive month of isolation were discarded. The resulting data set included a total 33,545 records. Data management procedures were performed using ACCESS, version 2002 (Microsoft, Redmond, USA).

The analysis was focussed on the six top reported serovars in the whole study period. The distribution of isolates over years was examined from 1980 to June 2012, whereas the distribution by sex, age group (<1, 1–5, 6–14, 15–64, and >65 years) and month of isolation (January–December) was examined using the 2000–June 2012 data set. Average annual isolation rates per 100,000 population were calculated by serovar, sex, age group, and province of residence standardised to the 2008 Italian reference population provided by the Italian National Institute of Statistics (ISTAT) (http://demo.istat.it/).

The inter-annual trend in the number of isolates from 2000 to 2011 was tested for statistical significance using the Cuzick's test for trend [12] (alpha level: 0.05). Data analysis was performed using EpiInfo2000, version 3.3.1 (CDC, Atlanta, USA), and STATA, version 11.2 (StataCorp, College Station, USA).

Shapefile of Italy with provincial administrative boundaries was obtained from the ISTAT (ED-1950-UTM coordinate system, zone 32 N). Average annual isolation rates per 100,000 population were presented using a choropleth map in ArcGis, version 9.0 (ESRI, Redlands, USA).

3. RESULTS

3.1. Inter-annual trends

From 1980 to June 2012, a total of 231,414 *Salmonella* isolates were reported.

The annual number of isolates decreased from 10,286 isolates on average per year in 1980–1995 to 3960 isolates on average per year in 1996–June 2012, with a more marked reduction from 2000 onwards (2,564 isolates on average per year).

During the whole study period, the top six reported serovars were *S.* Enteritidis (57,571 isolates; 24.8% of the total number of *Salmonella* isolates; average isolation rate: 3.04 isolates per 100,000 population/year), *S.* Typhimurium (56,969; 24.6%; 3.01 per 100,000/year), *S.* Infantis (10,134; 4.3%; 0.53 per 100,000/year), *S.* Derby (8,298; 3.5%; 0.46 per 100,000/year), *S.* 4,[5],12,:i:- (2,690; 1.2%; 0.47 per 100,000/year) and *S.* Napoli (883; 0.4%; 0.12 per 100,000/year). The other serovars accounted cumulatively for 94,869 isolates (41.2%; 5.01 per 100,000/year) (Figure 1).

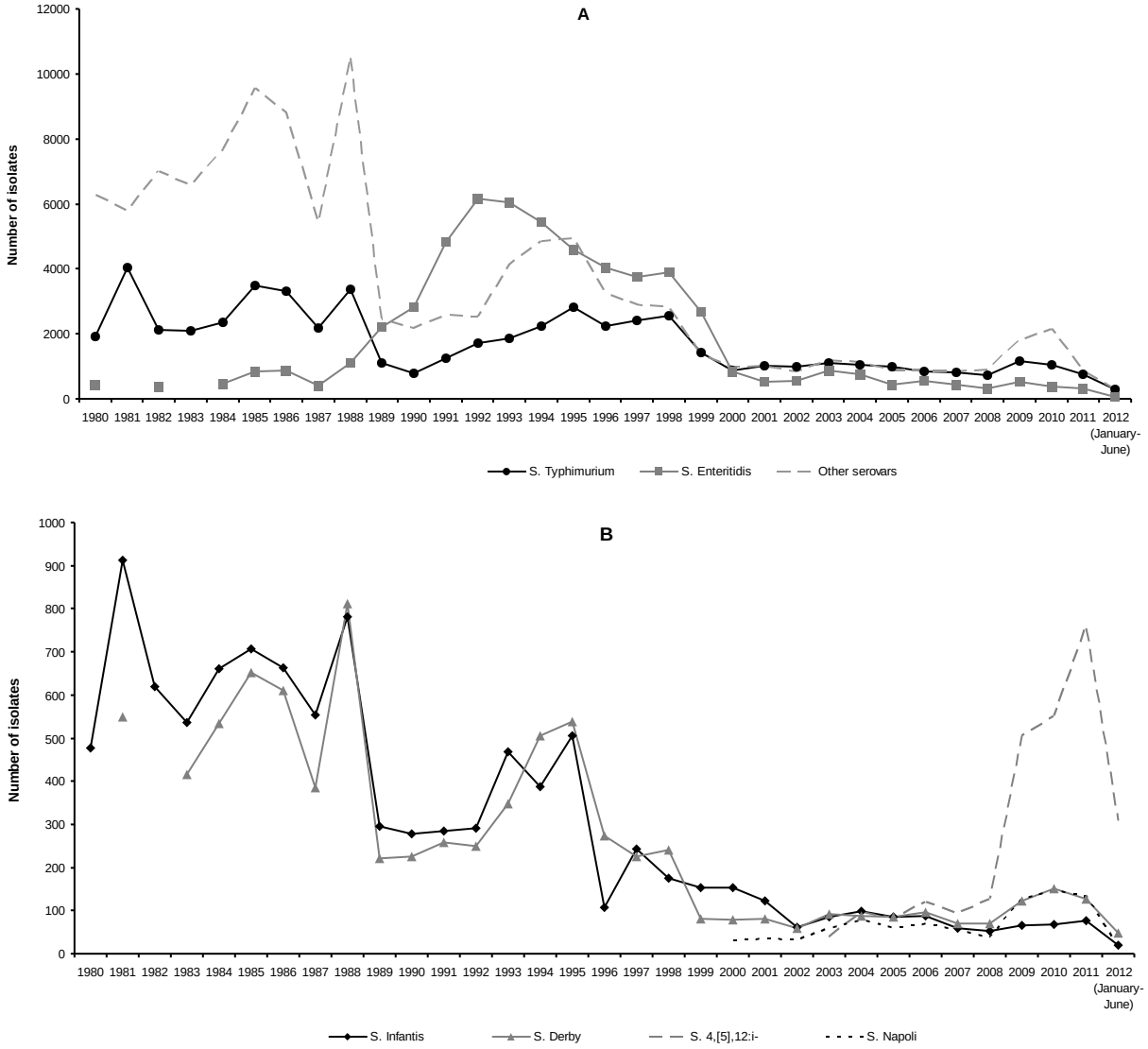

Figure 1. Temporal trend of the top six reported *Salmonella enterica* serovars in Italy from 1980 to June 2012: *S.* Typhimurium and *S.* Enteritidis (A); *S.* Infantis, *S.* Derby, *S.* 4,[5],12,:i:-, and *S.* Napoli (B). "Other serovars" in graph (A) include all serovars other than *S.* Typhimurium and *S.* Enteritidis.

S. Typhimurium was the predominant serovar from 1980 to 1988, but in 1989 *S.* Enteritidis overcame *S.* Typhimurium and dramatically increased in the following years, reaching a peak in 1992. Since then, *S.* Enteritidis started decreasing, and from 2000 onwards *S.* Typhimurium returned to be the predominant serovar (Figure 1).

S. Infantis alternated the position of the third most frequently reported serovar with *S.* Derby during the whole study period (Figure 1). However, while *S.* Infantis showed a marked decrease from 2002 onwards (<100 isolates per year), *S.* Derby increased since 2003, doubling the number of *S.* Infantis isolates in the last period (2009 to 2011).

In 2000 and 2003, *S.* Napoli and *S.* 4,[5],12,:i:- emerged, respectively. *S.* Napoli increased from 31 isolates in 2000 to 134 isolates in 2011. *S.* 4,[5],12,:i:- was isolated for

the first time in Italy in 2003 with 40 isolates (1.3% of the total number of isolates of that year). Since then, it increased steadily, reaching 762 isolates (39.1%) in 2011.

The decreasing trends observed from 2011 to 2012 (Figure 1) is due to the fact that the data for 2012 are partial, covering only the first six months of the year.

From 2000 to 2011, a significantly increasing temporal trend in the number of isolates was observed for *S.* Derby (+8.1% isolates on average per year, p<0.001; average isolation rate: 0.16 isolates per 100,000 population/year), *S.* Napoli (+28.2%, p = 0.032; 0.22 per 100,000/year) and *S.* 4,[5],12:i:- (+66.4%, p<0.001; 0.33 per 100,000/year), whereas a significantly decreasing temporal trend was observed for *S.* Infantis (–2.8%, p<0.001; 0.14 per 100,000/year) and *S.* Enteritidis (–3.0%, p<0.001; 0.91 per 100,000/year) isolates. *S.* Typhimurium isolates did not show any significant trend from 2000 to 2011 (p = 0.11; 1.58 per 100,000/year).

3.2. Seasonal distribution

The largest proportion of *Salmonella* isolates was observed in September (12.2%) and the smallest in February (6.0%). The mean number of isolates in these two months was 330 and 160 respectively (Figure 2). Although this seasonal pattern was consistent for most serovars, *S.* Napoli and *S.* Derby showed slight variations. *S.* Napoli increased steeply in June (9 isolates, on average) and peaked in July (14 isolates), remained at high levels from July to September (41 isolates) and then decreased rapidly in October (9 isolates). *S.* Derby peaked in September (11 isolates) but remained at a high level until October (11 isolates), with a slight decrease from November to March (41 isolates) (Figure 2).

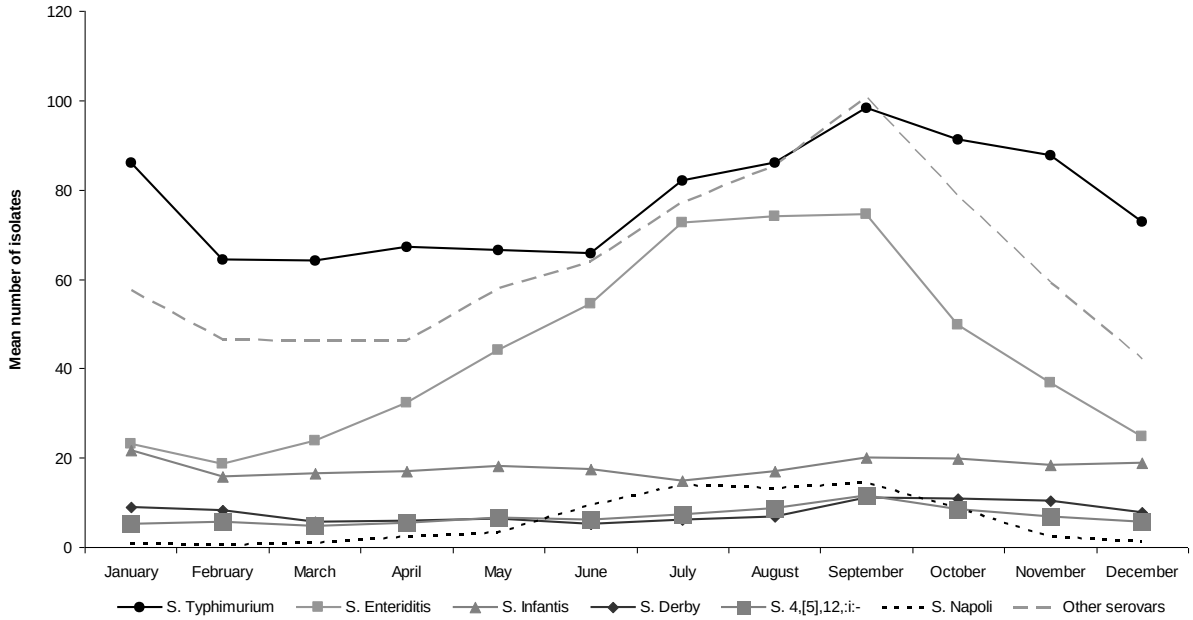

Figure 2. Average number of isolates of the top six reported *Salmonella enterica* serovars by month of isolation, Italy, 2000–mid 2012.

3.3. Age and sex distributions

During the 2000–mid 2012 period, the highest isolation rate was for children aged 1–5 years, at 32.37 isolates per 100,000 population/year, followed by children aged <1 year (13.69 per 100,000/year) and 6–14 years (7.98 per 100,000/year). In the other age groups, the average isolation rate was <3 isolates per 100,000/year. There were no evident differences in isolation rates between males and females (4.01 and 4.55 isolates per 100,000/year, respectively) (Table 1).

Table 1. Age and sex distribution of the annual isolation rate (number of isolates/100,000) of the top six reported *Salmonella enterica* serovars in Italy, 2000–June 2012.

Serovar	0–11 months	1–5 years	6–14 years	15–64 years	≥65 years	Female	Male
S. Typhimurium	3.89	14.07	3.18	0.42	0.77	1.40	1.69
S. Enteritidis	2.61	6.32	2.03	0.42	0.47	0.92	0.97
S. 4,[5],12:i:-	0.76	2.34	0.61	0.07	0.17	0.24	0.28
S. Derby	0.44	0.84	0.16	0.06	0.19	0.14	0.15
S. Infantis	0.36	0.67	0.17	0.06	0.13	0.13	0.15
S. Napoli	0.67	1.04	0.21	0.02	0.09	0.10	0.12
Other serovars	4.96	7.09	1.63	0.52	1.04	1.06	1.19
Overall	13.69	32.37	7.98	1.58	2.85	4.01	4.55

The 3.3% of isolates reported from 2000 to 2010 were from cases aged <1 year, 38.8% from cases aged 1–5 years, 17% from cases aged 6–14 years, 26.1% from cases aged 15–64 years, and 14.6% from cases aged ≥65 years.

Considering the top six reported serovars, *S.* Typhimurium showed the highest isolation rate in all age groups, particularly in children (where it accounted for 28% and 43% of isolates from children aged 1–5 and 6–14 years, respectively), but not in cases aged 15–64 years (where *S.* Typhimurium and *S.* Enteritidis accounted for almost the same proportion of isolates: ~27%). This is also evident for *S.* 4,[5],12:i:- that had a visibly higher isolation rate than *S.* Derby and *S.* Infantis in cases aged 1–5 years but not in cases aged 15–64 years, where *S.* 4,[5],12:i:-, *S.* Derby, and *S.* Infantis had almost the same isolation rate. Moreover, while *S.* Napoli was the fourth most isolated serovar in cases aged ≤14 years, it was the least represented in those aged >14 years.

3.4. Spatial distribution

Figure 3 presents the distribution at the province level of the average annual isolation rate per 100,000 population of the top six reported serovars (2000 to mid 2012). Except for the southern province of Isernia, the highest incidence rates were observed in the northern provinces of the country, particularly in the provinces of Sondrio, Trento, and Varese, whereas the southern provinces showed considerably lower incidence rates. Such spatial distribution was also observed in the incidence rate of the different serovars.

4. DISCUSSION

Evidence that human salmonellosis in Italy has decreased since the late 1990s has previously been provided through the analysis of cases notified to the SIMI [9]. This study showed that, since 2000, this decrease has concerned only specific serovars, namely *S.* Enteritidis and *S.* Infantis, whereas other serovars have emerged (*S.* 4,[5],12:i:-, *S.* Derby, and *S.* Napoli) or remained fairly stable (*S.* Typhimurium) over time.

After the global emergence of *S.* Enteritidis in the late 1980s that apparently filled the ecological niche vacated by the eradication of *S.* Gallinarum from poultry [13], a sustained decrease in the number of human *S.* Enteritidis infections has been observed in most countries since the late 1990s, e.g. [4,14–17]. Several factors, including the implementation of new on-farm control measures against *Salmonella* in poultry (e.g. the introduction of live vaccines), improved hygiene and education of consumers and food-workers, have probably contributed to this decrease [4,15]. Indeed, in 1992, the European Parliament issued a directive (Council Directive 92/117/EEC) establishing measures for protection against specified zoonotic agents in animals and foods of animal origin. This Directive proposed that the EU Member States establish monitoring systems and control measures in poultry breeding flocks. In 2003, to enforce these measures, the European Parliament and the EU Council introduced the Regulation No. 2160/2003 to ensure that proper and effective measures were undertaken to control *Salmonella* at all relevant stages of production, processing, and distribution. The observed decrease of *S.* Enteritidis suggests that these measures have succeeded in reducing the burden of human *S.* Enteritidis infection.

In Italy, however, we observed a peculiar profile of serovars, as *S.* Enteritidis fell consistently below *S.* Typhimurium since 2000, whereas in most other countries, despite the significant decrease of *S.* Enteritidis, *S.* Typhimurium has never become the most reported serovar, at least until the end of the 2000s [17]. This is particularly evident in the EU, where few countries in addition to Italy have recently experienced this shift in the dominant serovar, i.e. Belgium, France and

Denmark [4]. However, *S.* Typhimurium has been predicted to become the most common serovar in England and Wales by 2012 as a result of the decrease of *S.* Enteritidis [18].

Given the distribution of serovars from humans and animal sources in the period 2007-2009, it has been estimated that pig is the most important source of human salmonellosis in Italy, accounting for 73% of human infections [4]. This is in line with our results, as pig is in fact the most important reservoir of *S.* Typhimurium [4].

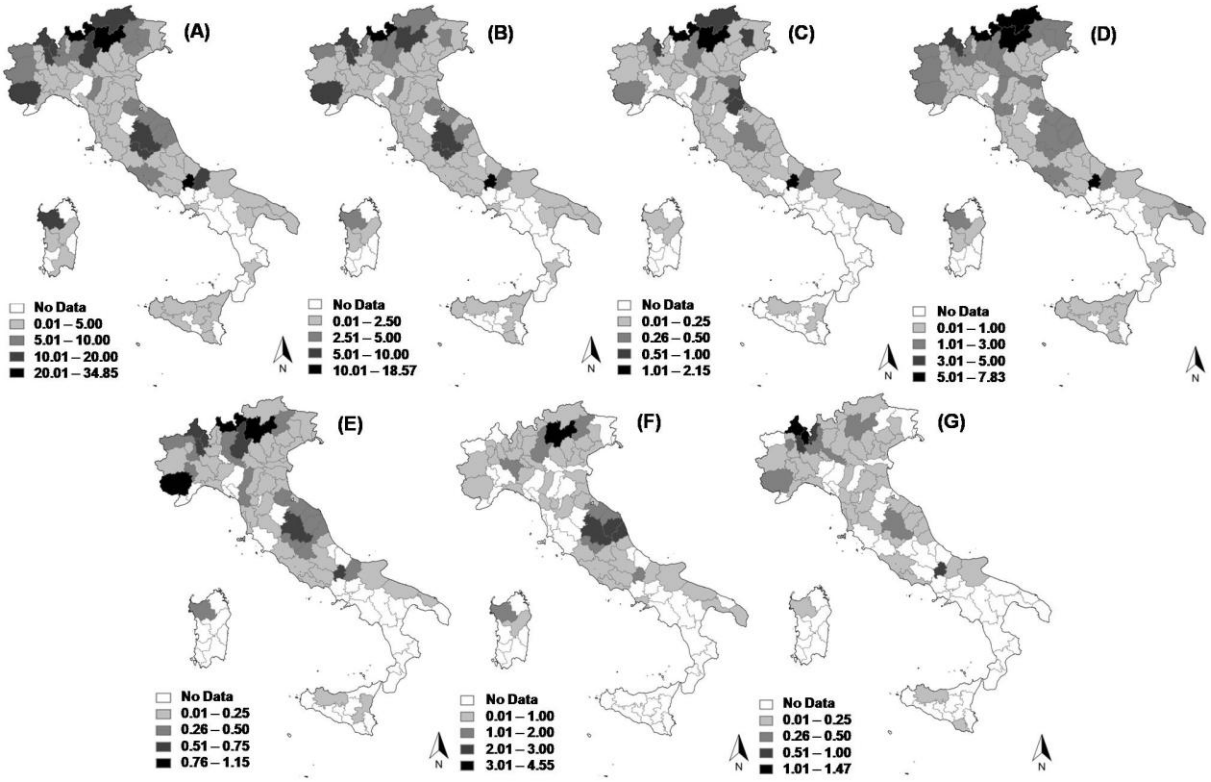

Figure 3. Maps (province level of detail) of the average annual isolation rates per 100,000 population of *Salmonella* in Italy in 2000-mid 2012 (all *Salmonella* isolates (A), *S.* Typhimurium (B), *S.* Infantis (C), *S.* Enteritidis (D), *S.* Derby (E), *S.* 4,[5],12:i:- (F), *S.* Napoli (G)).

As laying hens are the most likely source of human *S.* Enteritidis infection in Europe [4], the drastic decrease of *S.* Enteritidis in Italy may be explained, to some extent, by the structure of the Italian poultry industry (that is highly integrated and vertically developed) and by the fact that poultry meat and table egg production in Italy is self-sufficient to meet the internal market demand. Moreover, since 2003, the level of biosecurity and hygiene practices in the Italian poultry industry have greatly been enhanced to address the legal requirements provided for the control of avian influenza epidemics [19]. These structural characteristics may have had a particularly significant impact on the effectiveness of the applied control measures against *S.* Enteritidis in the Italian poultry industry, as both the production and consumption of poultry products is vertical and rather closed to external influences.

The monophasic variant of *S.* Typhimurium, *S.* 4,[5],12:i:-, characterised by the antimicrobial resistance to Ampicillin, Streptomycin, Sulphonamide, and Tetracycline (pattern ASSuT) is emerging and extensively circulating in Italy, Denmark, and the UK [11,20]. In Italy, *S.* 4,[5],12:i:-, showed a dramatic increase since 2003, both in humans and in food-producing animals, particularly pigs and bovines [21]. Also *S.* Napoli is an emerging serovar in Europe, with most of the cases (87%) occurring in Italy, France, and Switzerland. It has been suggested that the environment can act as the main reservoir for *S.* Napoli, and from there it can spill over to animals and humans [10].

Most serovars showed a marked seasonality, increasing over the summer months and peaking in August/September, and then decreasing gradually. Although the reasons of this pattern are not entirely known, it may be related to the parallel *Salmonella* shedding trend in animal hosts, insufficient refrigeration and mishandling of foods during the warm months [22,23].

As expected, isolation rates were highest in children. This may be due to the

greater proportion of symptomatic infections amongst the young but also to the higher propensity to take samples by paediatricians (i.e. detection bias) [23]. However, consistent with other studies [10,11,23], we observed that cases with *S*. Typhimurium, *S*. 4,[5],12:i:-, or *S*. Napoli infection were most likely to be children, whereas cases with *S*. Enteritidis, *S*. Derby, or *S*. Infantis infections were more likely to be adults. This may be due to the different serovar-specific risk factors to which individuals are exposed at varying age groups [24].

This study is based on reported data from laboratories that are not homogenously distributed in the Italian territory; thus, there may be differences in representativeness of the data from different regions. It has been showed that the surveillance systems of northern regions of Italy are generally more sensitive in detecting cases of infectious gastroenteritis, leading to significantly higher notification rates of salmonellosis compared to the national average [9]. Moreover, diagnostic capacity for enteropathogens differs from laboratory to laboratory in Italy [25]. These may be the reasons as to why we observed that the isolation rates were considerably lower in the southern part of the country.

With regard the selection of isolates included in our analyses we deleted duplicates but we cannot avoid including outbreak-related cases because epidemiological information on the origin of the isolates were not available. This condition could have biased the relative percentages of the *Salmonella* serovars in case of relevant outbreaks.

In conclusion, *Salmonella* serotyping is useful for informing and addressing public health actions, providing data about the emerging serovars (which may reveal the presence of a previously unrecognised source of infection) and the efficacy of intervention measures.

We found that *S*. Enteritidis has decreased dramatically in Italy and that *S*. Typhimurium has become again the most reported serovar as from 2000. It is noteworthy that while *S*. Enteritidis and *S*. Infantis decreased, *S*. Typhimurium remained stable and *S*. 4,[5],12:i:-, *S*. Derby, and *S*. Napoli increased. This suggests that the applied control measures are not equally efficient against the considered serovars and that other sources of infection have probably become increasingly important (e.g. unconventional, wild and free-rage animals, fruit and vegetables, etc.). Therefore, further investigation into potential causes of the spread of the emerging serovars against which newly tailored control measures should be implemented is warranted.

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Chapter 4

Attribution of human *Salmonella* infections to animal and food sources in Italy (2002–2010): adaptations of the Dutch and modified Hald source attribution models

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Attribution of human *Salmonella* infections to animal and food sources in Italy (2002–2010): adaptations of the Dutch and modified Hald source attribution models

ABSTRACT

The Dutch and modified Hald source attribution models were adapted to Italian Salmonella data to attribute human infections caused by the top 30 serotypes between 2002 and 2010 to four putative sources (*Gallus gallus*, turkeys, pigs and ruminants), at the points of animal reservoir (farm), exposure (food), and both combined. Attribution estimates were thus compared between different models, time periods and sampling points. All models identified pigs as the main source of human salmonellosis in Italy, accounting for 43–60% of infections, followed by *Gallus gallus* (18–34%). Attributions to turkeys and ruminants were minor. An increasing temporal trend in attributions to pigs and a decreasing one in those to *Gallus gallus* were also observed. Although the outcomes of the two models applied at farm and food levels essentially agree, they can be refined once more information will become available, providing valuable insights about potential targets along the production chain..

1. INTRODUCTION

Salmonellosis is a major cause of human bacterial gastroenteritis and the second most reported food-borne zoonosis in the European Union (EU), after campylobacteriosis [1]. Humans can become infected with *Salmonella* from several sources and via different pathways, including direct contact with live animals, environmental and, to a lesser extent, anthroponotic transmission. However, the most common source is by far contaminated food, with 86–95% of cases estimated to be food-borne [2,3]. In recent years, human cases of salmonellosis reported by Italian general practitioners have decreased spectacularly, passing from 47 to 7 cases per 100,000 population in less than two decades [4]. This decrease has mainly concerned infections with *S*. Enteritidis, while infections with other serotypes have increased (e.g., *S*. Typhimurium monophasic variant 4,[5],12:i:- and *S*. Derby) or have remained fairly stable (e.g., *S*. Typhimurium) [5], suggesting that the relative importance of the different sources of human salmonellosis has changed over time.

Attributing human *Salmonella* infections to specific sources is crucial to prioritize and implement targeted interventions in the food chain, as well as to measure the impact of such interventions [6]. The term "source" is often used as a collective term to cover any point along the transmission pathway, such as the animal reservoirs or amplifying hosts (e.g., chicken, cattle, pig, etc.), the vehicles or exposures (e.g., food, water, direct contact with animals, etc.) and even specific food items (e.g., pork, milk, eggs, etc.). Several methods have been proposed for source attribution of food-borne diseases [7,8]. In particular, the microbial subtyping approach, based on the comparison of the frequency distributions of pathogen subtypes isolated from humans with those isolated predominantly from putative animal, food and environmental sources, has received considerable attention since the development of the Hald model for *Salmonella* source attribution in Denmark [9]. The Hald model, a Bayesian adaptation of the earlier frequentist Dutch model [10], attributes stochastically human *Salmonella* infections to each putative source, to travelling abroad and to outbreaks, while accounting for differences among the different *Salmonella* subtypes and sources to cause human infection [9]. The Hald model has successfully been adapted to salmonellosis in several countries [6,11–15]. Yet, to further improve its identifiability and to handle with uncertainty in data of poorer quality, a

modified Hald model has also been proposed [16].

While the Dutch model uses a straightforward approach, providing transparent insights into the functionality of the attribution process [15], the Hald model is a more complex model that fits parameters with no clear biological interpretation, therefore considered a sort of "black box" model [11]. So far, these two models have been applied to single points of the farm-to-fork continuum only, e.g. point of reservoir, point of exposure, or both combined (undifferentiated). The comparative application of these two models to different points of attribution may further inform us about the most promising targets on which risk management strategies should be focused.

The main aim of this study was to adapt the Dutch and Hald source attribution models to Italian *Salmonella* data in order to estimate the proportions of domestic, sporadic human *Salmonella* infections in Italy attributable to four putative sources (*Gallus gallus*, turkeys, pigs and ruminants), which have been monitored for a period of nine years (2002–2010) both in animals and in foods of animal origin. Moreover, we explored the extent to which the comparison of attribution estimates between the point of farm and that of food is useful in informing risk managers.

2. MATERIALS AND METHODS

2.1. Laboratory surveillance of *Salmonella* in humans

In Italy, testing for *Salmonella* infection is usually performed on patients with gastroenteritis seeking for medical care or on people requiring periodic testing regardless of symptoms (e.g., food handlers, healthcare workers, etc.). Irrespective of symptomatology, *Salmonella* isolates from humans are reported to Enter-net Italia, a passive, laboratory-based surveillance system for human enteropathogens based on a network of more than 140 peripheral laboratories with approximately 65% coverage of Italian territory, concentrated mainly on the northern part of the country. Enter-net Italia is complementary to the Italian National Surveillance System for Infectious Diseases (SIMI) [17]. From the peripheral laboratories, Enter-net Italia collects demographic and microbiological information (at least the serotype) on *Salmonella* isolates of ~50% of human cases of salmonellosis notified to the SIMI [18]. Information on travel history or link to outbreaks concerns approximately 15% of serotyped isolates. At present, *Salmonella* isolates reported to Enter-net Italia are virtually indistinguishable between symptomatic and asymptomatic human infections. For the purposes of this study, a human *Salmonella* infection was considered to be: 1) travel-related if the person has travelled abroad during the incubation period; and 2) outbreak-related if the person has had contacts with people with gastroenteritis and/or there have been other epidemiologically-linked infections.

2.2. Veterinary surveillance of *Salmonella*

Findings of *Salmonella* in animals and foods of animal origin as part of diagnostic or monitoring activities are notifiable to Italian veterinary authorities. All major food-producing animals and foods of animal origin in Italy are tested for *Salmonella* according to official control programmes (Directive 2003/99/EC, Regulations EC 2160/2003 and 882/2004). Positive samples are reported to Enter-vet, the Italian veterinary surveillance system for *Salmonella*. Enter-vet was established in 2002 and is based on a network of 10 peripheral laboratories covering the whole country through the regionally competent Institutes for Animal Health (Istituti Zooprofilattici Sperimentali). Approximately 5000 *Salmonella* serotyped isolates from animals and foods of animal origin are reported to Enter-vet each year and classified by animal species and sampling point (farm or food).

2.3. *Salmonella* data included in the models

The input dataset for the *Salmonella* attribution models included surveillance data over nine years (from January 2002 to December 2010) collected by Enter-net and Enter-vet. Based on the most frequently isolated *Salmonella* serotypes from humans in common with at least one of the sources, the following 30 serotypes were included in the models: Typhimurium and its monophasic variant 4,[5],12:i:-, Enteritidis, Derby, Infantis, Muenchen, Hadar, London, Bredeney, Brandenburg, Rissen, Panama, Thompson, Virchow, Goldcoast, Give, Blockley, Newport, Heidelberg, Agona, Anatum, Saintpaul, Coeln, Montevideo, Kapemba, Mbandaka, Kedougou,

Meleagridis, Senftenberg and Livingstone. The selected serotypes accounted for 20890 human infections, corresponding to 87% of all human *Salmonella* infections reported in the study period. The remaining 13% of human infections caused by less frequent serotypes were excluded from the models and were not further considered in this study. A closer look at the data revealed that the excluded infections were often associated with travel and their serotypes were rarely, if ever, detected in the considered sources. Duplicate entries, i.e. different *Salmonella* isolates from a same person because of the follow-up of people with *Salmonella* infection after the first isolation, were discarded. Therefore, the models attributed only those human *Salmonella* infections that, during the entire study period and irrespective of clinical manifestations, were caused by the aforementioned top 30 *Salmonella* serotypes found both in humans and in the considered animal and food sources.

Frequencies of human infections were merged with the animal and food isolates by serotype, sampling point and year. Based on data availability, the following sources were considered: *Gallus gallus*, turkeys, pigs, and ruminants (cattle, sheep and goats, combined). These sources were consistently sampled at the level of farm (live animals) and at that of retail (food of animal origin) during the entire study period. Differentiation of *Gallus gallus* between broilers and layers/eggs was not possible because the data were available at the species level only.

To avoid sparse data that may lead to a low precision of the serotype prevalence estimates [16], the merged dataset was arranged in three 3-year periods (2002–2004, 2005–2007 and 2008–2010) . The resolution of phage typing data was very low and did not allow for the use of this information in the analysis. Serotype frequencies in humans and in animal and food sources are reported in Table 1.

2.4. Overview of the models

A modified version of the Dutch model and a Hald model accommodating for temporal dimension [11] with some further adjustments as proposed by Mullner *et al*. [16] were developed to estimate the proportions of domestic, sporadic human *Salmonella* infections in Italy attributable to the four putative sources at farm (reservoir) level, at food (exposure) level, and at both these levels combined. Domestic and sporadic infections are defined as infections acquired in Italy and not implicated in outbreaks.

Where the 95% credible intervals (CIs) of the attribution estimates did not overlap each other, these were considered to be significantly different from one another at the 5% level of significance.

2.4.1. Modified Dutch model

The original Dutch model compares the number of human *Salmonella* infections caused by a particular serotype with the relative occurrence of that serotype in each source [10]. The expected number of human infections (λ_{ijt}) caused by serotype i from source j in period t is given by:

$$\lambda_{ijt} = \frac{r_{ijt}}{\sum_j r_{ijt}} \times e_{it}$$

where r_{ijt} is the relative occurrence of serotype i from source j in period t, and e_{it} is the estimated number of sporadic and domestic human infections of serotype i in period t (see Table 2 for notations and estimation of e_{it}). A sum over serotypes gives the total number of infections expected from source j in period t, denoted by:

$$\lambda_{jt} = \sum_i \lambda_{ijt}$$

In this study, the Dutch model was modified to incorporate prevalence uncertainty and food consumption weights. Prevalence was modelled using the novel approach proposed by Mullner *et al*. [16] based on the assumption that $p_{ijt} = \pi_j \times r_{ijt}$, where p_{ijt} is the prevalence of serotype i from source j in period t, π_j is the overall prevalence of all *Salmonella* serotypes in source j, and r_{ijt} is the relative occurrence of serotype i from source j in period t. Uncertainty was introduced in the estimates of the prevalence by assuming the following probability distributions:

$$\left(r_{1jt}, r_{2jt}, \ldots, 1-\sum_{i=1}^{I-1} r_{ijt}\right) \sim Dirichlet\,(X_{1jt}, X_{2jt}, \ldots, X_{Ijt})$$

where X_{ijt} (with $i = 1, 2, \ldots, I$) are the source isolates of serotypes i from source j at time t, and

$$\pi_j \sim Beta\ (\alpha_j+1, \beta_j+1),$$

where α_j are the *Salmonella*-positive sampling units from source j and $\beta_j = N - \alpha_j$, with N being the total number of sampling units from source j that have been tested for *Salmonella* spp. The number of tested sampling units and respective positivity percentages in different animal reservoirs in Italy were provided by Pires *et al*. [14] by collating available information from the EU *Salmonella* prevalence baseline survey and from the EU Summary Reports on Trends and Sources of Zoonoses, Zoonotic Agents and Food-Borne Outbreaks, as published annually by the European Food Safety Authority from 2006 to 2009. These data were provided at animal/sample level for broilers, bovines and pigs, and at flock/herd level for layers and turkeys.

Average per capita daily food consumption (g/person/day) for source j in period t in Italy, denoted as m_{jt}, was obtained from the Eurostat database (http://epp.eurostat.ec.europa.eu/portal/page/portal/food/data/database) for ruminant and pig meats. As the Eurostat database provides data on poultry consumption as a whole with no differentiation between *Gallus gallus* (meat/eggs) and turkey, we used the data from the National Association of Poultry Producers (http://www.unionenazionaleavicoltura.it/prodcons.aspx). Uncertainty was introduced in the estimates of m_{jt} by assuming that $\log(m_{jt}) \sim \text{Normal}(\mu_{jt}, \sigma_{jt})$, where μ_{jt} and σ_{jt} are respectively the mean and standard deviation of the per capita daily food consumption for source j in period t. Using the above notations and those in Table 2, the modified Dutch model we used is denoted by:

$$\lambda_{ijt} = \frac{p_{ijt} \times m_{jt}}{\sum_j p_{ijt} \times m_{jt}} \times e_{it}$$

The model was implemented in @Risk by setting 100000 iterations with the Latin hypercube sampling technique and a seed of 1.

2.4.2. Modified Hald model

The Hald model compares the number of human infections caused by different serotypes with their prevalence in the different sources, accounting for the amount of food consumed and incorporating serotype- and source-dependent factors [9]. By using a Bayesian approach, this model can explicitly incorporate prior information and quantify the uncertainty around each of the parameters. We applied the modified version of the Hald model as described elsewhere [16]. Using the above notations and those reported in Table 2, we assumed that

$$o_{it} \sim Poisson(\sum_j \lambda_{ijt})$$

and that

$$\lambda_{ijt} = m_{jt} \times p_{ijt} \times q_i \times a_j$$

where o_{it} is assumed to be Poisson distributed; p_{ijt} was modelled using the aforementioned novel approach of Mullner *et al*. [16]; q_i is the serotype-dependent factor, which putatively accounts for differences in survivability, virulence and pathogenicity of serotypes i; and a_j is the source-dependent factor, which putatively accounts for the ability of the sources j to act as vehicles for *Salmonella* (e.g., differences in pathogen load, source characteristics influencing pathogen growth, preparation/handling procedures, differences in sensitivity of surveillance programmes and randomness of sampling schemes).

In accordance with Mullner *et al*. [16], both q_i and a_i were assumed to be constant over time and q_i was modelled hierarchically as $\log(q_i) \sim \text{Normal}(0, \tau)$, where τ is given by a fairly diffuse Gamma(0.01, 0.01) distribution. Parameter a_j was defined as uninformative Uniform(0, 100) distribution. Parameter q_i for *S*. Typhimurium monophasic variant 4,[5],12:i:- was set to be equal to that of *S*. Typhimurium. Yet, exploratory analyses revealed that setting different q_i parameters for *S*. Typhimurium and its monophasic variant 4,[5],12:i:- had no influence on model results.

Posterior distribution was obtained by a Markov Chain Monte Carlo simulation implemented in WinBUGS 1.4. Five independent Markov chains were run for 30,000 iterations after a burn-in period of 10,000 iterations, which proved able to provide convergence as monitored by the method developed by Gelman and Rubin [19].

Table 1. Frequencies of *Salmonella* serotypes isolated from humans and from animal and food sources, at farm and food level, in (I) 2002-2004, (II) 2005-2007, and (III) 2008-2010, Italy.

Serotype	Humans			*Gallus gallus*						Pigs						Turkeys						Ruminants					
				Farm			Food			Farm			Food			Farm			Food			Farm			Food		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Typhimurium	3140	2667	2919	129	188	161	45	274	35	456	535	371	796	502	398	108	195	10	73	60	52	199	160	175	90	112	77
4,[5],12:i:-	136	300	1324	9	9	74	6	88	22	138	263	817	106	175	609	4	11	16	4	5	24	5	28	100	4	12	35
Enteritidis	2181	1453	1212	159	244	377	167	100	82	1	16	3	10	159	8	1	1	6	1	5	28	3	5	6	11	6	20
Derby	239	253	344	5	6	8	16	159	17	159	359	164	577	310	331	26	14	4	20	8	10	6	16	22	26	12	13
Infantis	245	232	185	40	31	60	47	30	23	6	23	41	99	63	32	1	1	0	0	0	12	1	2	4	2	1	9
Muenchen	144	67	145	0	24	193	2	5	44	0	2	14	22	19	3	0	1	0	1	1	10	0	0	3	0	4	2
Hadar	141	60	127	187	148	224	215	50	93	3	8	2	12	48	2	59	16	7	46	19	41	6	0	1	9	3	2
Rissen	54	52	124	0	6	13	10	32	5	0	85	46	77	76	93	0	1	0	0	0	0	0	2	1	4	5	30
London	103	61	103	8	0	5	32	39	4	9	36	61	139	66	67	1	0	0	1	0	0	3	2	5	8	0	9
Bredeney	108	60	96	0	0	0	25	53	90	0	0	0	124	116	24	0	0	0	10	41	31	0	0	0	12	7	5
Newport	36	41	96	0	0	15	0	0	12	0	0	1	0	0	5	0	0	74	0	0	95	0	0	9	0	0	2
Goldcoast	74	30	89	0	0	0	0	0	0	0	0	0	37	0	0	0	0	0	1	0	0	0	0	0	1	0	0
Brandenburg	97	58	84	132	101	161	1	1	0	53	44	26	67	27	0	3	103	22	0	0	0	6	22	4	1	1	0
Give	44	71	74	3	0	1	0	29	0	2	19	11	17	0	14	0	0	0	1	0	0	15	1	7	0	3	0
Panama	110	40	72	0	0	0	0	0	0	0	0	0	46	0	0	0	0	0	0	0	0	0	0	0	4	0	0
Thompson	79	68	62	63	50	186	29	24	19	1	11	2	1	65	1	1	1	0	0	1	0	5	1	8	3	0	2
Coeln	11	15	58	0	2	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16	0	0	0	0	0	0
Agona	61	30	50	13	13	61	27	14	0	0	4	3	19	10	0	36	26	1	22	34	0	1	0	1	5	8	0
Saintpaul	60	22	48	5	2	0	50	1	19	0	1	0	6	1	6	13	22	0	39	6	58	1	1	0	10	1	4
Virchow	89	60	46	256	135	0	68	1	0	4	0	0	4	51	0	1	0	0	1	0	0	4	0	0	1	2	0
Anatum	54	44	41	10	3	14	8	73	0	67	54	21	123	40	0	33	4	0	41	7	0	3	4	3	14	4	0
Livingstone	71	47	39	0	0	0	129	93	21	0	0	0	73	192	5	0	0	0	0	2	9	0	0	0	2	3	1
Kapemba	9	13	38	0	0	0	0	0	0	0	0	0	0	0	18	0	0	0	0	0	0	0	0	0	0	0	4
Blockley	118	34	22	62	34	13	56	26	0	9	1	1	8	21	0	148	40	5	99	27	0	8	3	1	9	1	0
Montevideo	24	27	21	0	42	110	9	0	45	0	1	0	1	0	7	0	0	5	1	0	0	0	0	2	1	0	1
Heidelberg	118	27	18	109	42	96	64	16	0	8	8	0	5	37	0	92	143	1	45	115	0	1	3	0	4	4	0
Mbandaka	10	3	12	0	37	129	0	15	0	0	1	0	0	58	0	0	0	0	0	1	0	0	2	0	0	1	1
Kedougou	9	2	9	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0
Meleagridis	9	4	5	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Senftenberg	5	5	2	23	19	22	12	0	3	0	1	0	3	0	0	2	1	1	0	0	3	0	0	2	1	0	2
Total	7579	5846	7465	1213	1136	1935	1018	1123	535	916	1472	1584	2372	2036	1626	529	580	152	406	332	390	267	252	354	222	190	219

Table 2. Parameters used to estimate the number of domestic and sporadic human *Salmonella* infections attributable to the animal and food sources

Notation	Description	Estimation
i	*Salmonella* serotype (30 serotypes)	Data
j	Animal or food source (4 sources)	Data
t	3-year period (2002-2004, 2005-2007, 2008-2010)	Data
o_{it}	Observed infections with serotype i in period t	Data
oyt_{it}	Observed infections with serotype i in period t reporting to have travelled abroad in the incubation period	Data
ont_{it}	Observed infections with serotype i in period t reporting to have not travelled abroad in the incubation period	Data
out_{it}	Observed infections with serotype i in period t with unknown travel history	Data
pt_{it}	Probability that a person infected with serotype i in period t with unknown travel history did travel	Beta(oyt_{it} + 1, ont_{it} + 1)
et_{it}	Estimated number of additional infections with serotype i in period t that had travelled	Binomial(out_{it}, pt_{it})
dc_{it}	Estimated total number of domestic infections with serotype i in period t	$o_{it} - oyt_{it} - et_{it}$
oyb_{it}	Observed infections with serotype i in period t known to be outbreak-related	Data
oub_{it}	Observed infections with serotype i in period t with no information on relationships with outbreaks	Data
pb_{it}	Probability that a person infected with serotype i in period t is outbreak-related	Beta(oyb_{it} + 1, $out_{it} - oyb_{it}$ + 1)
eb_{it}	Estimated number of additional domestic infections with serotype i in period t that are outbreak-related	Binomial(dc_{it}, pb_{it})
e_{it}	Estimated total number of domestic and sporadic infections with serotype i in period t	$dc_{it} - oyb_{it} - eb_{it}$

3. RESULTS

3.1. Modified Dutch model

Mean percentages and respective 95% CIs of human *Salmonella* infections attributed to each of the sources, to travelling abroad and to outbreaks by the modified Dutch model are reported by time period in Figure 1. Overall (2002–2010), pigs were the source causing the highest percentage of human *Salmonella* infections attributed at the levels of farm, i.e. animals (43%, 95% CI: 42–44%), food (45%, 44–46%) and both combined (44%, 43–45%), followed by *Gallus gallus* (farm: 34%, 32–35%; food; 32%, 31–33%; farm + food: 33%, 32–34%), turkey (4%, 4–5% at all levels) and ruminants (2%, 2–3% at all levels). Infections estimated to be travel- and outbreak-related amounted to 16% (15–17%) and 1% (1–1%), respectively.

A significant decrease in the percentage of infections attributed to *Gallus gallus* was observed from 2002–2004 to 2008–2010 (–6%, –4% and –4%, on average, per each 3-year period in animals, food and both combined, respectively), whereas the percentage of infections attributed to pigs increased significantly (+4%, +2% and +3%, on average, per each 3-year period in animals, food and both combined, respectively). Percentages of infections attributed to the other sources, to travelling abroad and to outbreaks did not vary significantly over time (Figure 1).

3.2. Modified Hald model

Percentages of human *Salmonella* infections attributed to each of the sources, to travelling abroad and to outbreaks by the modified Hald model are reported by time period in Figure 1. Pigs were again the source that accounted for the highest percentage of infections attributed to animals (60%, 95% CI: 48–72%), food (47%, 41–52%) and both combined (47%, 42–52%), followed by *Gallus gallus* (farm: 18 %, 4–31%; food: 33%, 28–38%; farm + food: 32%, 27–37%). Turkeys were the third most important source at farm level (3%, 0–7%) and at both farm and food levels combined (2%, 0–5%), but it was the fourth at food level (1%, 0–4%), behind ruminants (farm: 2%, 0–5%; food: 0–3%; farm + food: 0–3%). Infections estimated to be travel- and outbreak-related amounted to 16% (15–17%) and 1% (1–1%), respectively.

From 2002–2004 to 2008–2010, percentages of infections attributed to *Gallus gallus* decreased by –4% (animals), –5% (food) and –5% (both animals and food combined), on average, per each 3-year period, whereas those attributed to pigs increased by +2% (animals), +2% (food) and +4% (both animals and food combined). However, none of these trends was significant as the CIs of attribution estimates were largely overlapping. Percentage of cases attributed to the other sources, to travelling abroad and to outbreaks did not vary significantly over time (Figure 1).

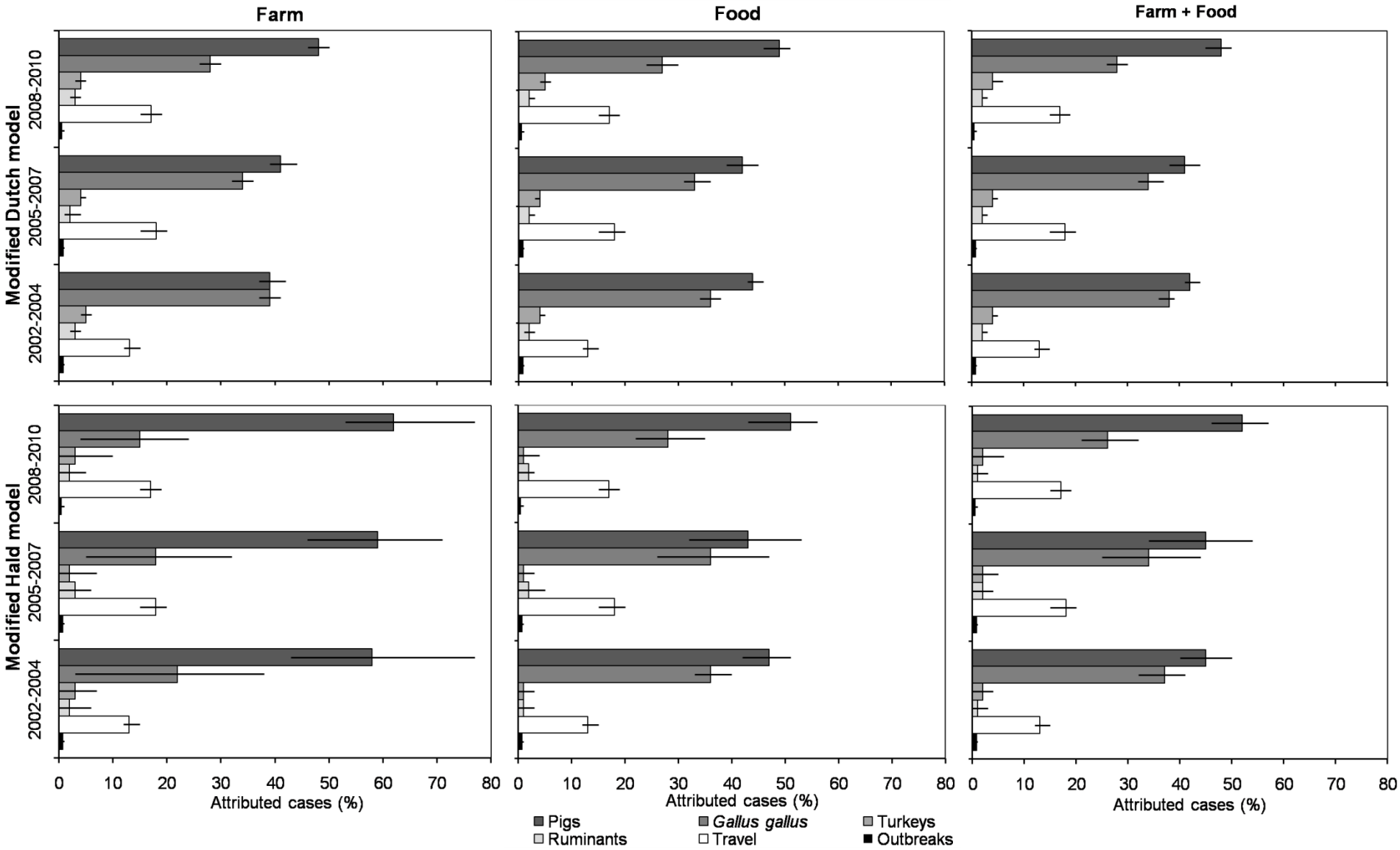

Figure 1. Percentages of human salmonellosis cases attributed to each putative source at farm and/or food level estimated by the modified Dutch and Hald models.

4. DISCUSSION

In this study, two models were developed to attribute domestic and sporadic human *Salmonella* infections caused by the 30 most frequently reported serotypes in Italy between 2002 and 2010 to four putative sources at the points of reservoir (food-producing animals), exposure (foods of animal origin), and both combined. This allowed us to compare the obtained attribution estimates between different models, sampling points and time periods.

Pigs stood out as the largest contributors to human salmonellosis in Italy, being responsible for about half of the infections relative to the other sources, to travelling abroad and to the outbreaks during the entire study period. This finding was consistent over different models, time periods and sampling points, and it was also in line with previous estimates based on the (original) Hald model applied to a rather different input dataset in which 73% of human *Salmonella* infections that occurred in Italy between 2007 and 2009 had indeed been attributed to pigs [14].

Besides Italy, another seven (out of 24) European countries considered by Pires *et al*. [14] have identified pigs as the most important source of human salmonellosis. These included Belgium, Cyprus, Finland, France, Ireland, Poland and Sweden, with very similar proportions of infections attributed to poultry and to pigs in the Netherlands [14]. Also in New Zealand pigs have been identified as the major source of human salmonellosis, accounting for 60% of infections, followed by poultry [16]. It is therefore becoming increasingly difficult to ignore that pigs play a paramount role as source of human salmonellosis, at least in the EU, and that (mis)handling and consumption of contaminated pork is the most likely food-borne pathway involved.

We observed an increasing temporal trend in the percentages of infections attributed to pigs and a concurrent reduction of those attributed to *Gallus gallus*. The decreasing importance of *Gallus gallus* is mainly driven by the drastic decrease in the number of human *S*. Enteritidis infections (Table 1), for which *Gallus gallus*, and particularly layers, are the major reservoir [14,20]. Such decrease has been observed in most European countries, including Italy, since the late 1990s as a result of the implementation of new on-farm control measures in poultry (e.g. introduction of live vaccines), improved hygiene, and education of consumers and food workers [14,21–24], especially after the implementation of national control programmes for *Salmonella* in poultry according to EU Regulation (EC) No. 2160/2003. Conversely, the increasing importance of pigs is mainly driven by the predominance of human infections caused by *S*. Typhimurium and its monophasic variant 4,[5],12:i:-, as well as by the increase of those caused by *S*. Derby (Table 1). Indeed, pigs are the most likely reservoir for *S*. Typhimurium, its monophasic variant 4,[5],12:i:- and *S*. Derby [14,20], and since 2000 in Italy, human *S.* Enteritidis infections fell consistently below those caused by *S*. Typhimurium, which has therefore become the most frequently isolated serotype from humans [5].

In all periods and sampling points, the two models have identified turkeys and ruminants as minor sources, accounting for 1–5% of human *Salmonella* infections. This is in line with previous estimates indicating that ~3% of all human *Salmonella* infections in the EU are attributable to turkeys relative to broilers, layers and pigs [6]. Ruminants have seldom been included as a putative source in attribution studies conducted in the EU, mainly because of data availability issues [14]. Although ground beef seems to be an important source of human salmonellosis in the US [13], there is also some evidence that ruminants do not play such a significant role [11,12,16].

Both our model adaptations retained much of the original methodology. Modelling the prevalence using the methodology of Mullner *et al*. [16] allowed us to take into account the overall probability of finding *Salmonella* in a given source (parameter π_j) in addition to the relative frequency of the different serotypes within each source (parameter r_{ijt}, reflecting our best guess of the within-source serotype probability distribution). This is a necessary step towards compensating the absence of intensive surveillance data for all relevant sources as required by the original Hald model [9]. Moreover, uncertainty around such estimates could not be ignored without overestimating the level of precision [16]. Therefore, by incorporating this additional stratum of information and uncertainty, the model can now make use of the best possible estimate of the prevalence. Nevertheless, concerns remain about the adequacy of the priors used for

modelling the parameter π_j, as these originated from both individual- and flock-level sampling schemes [14] and did not change either over attribution points or over time periods. This implied that the overall probability of finding *Salmonella* in a given source, as expressed by pooling the available data at different resolutions, was assumed to be a property of the sources themselves and to be relatively stable over time and along the farm-to-food continuum. Changes in the prevalence were therefore primarily due to changes in the within-source serotype distribution.

As reliable food consumption data were available and environmental, anthroponotic or unknown sources were not included in the models, food consumption weights were incorporated to take into account the human exposure to the different sources. The importance for human *Salmonella* infection of food-borne exposure is unquestionable [2,3]; thus, by incorporating food consumption data the models are better informed and can more closely reflect the chance of a given source to act as a vehicle for *Salmonella*. This incorporation is particularly relevant in the modified Dutch model because this model no longer assumes that within each subtype the impact of the different sources is equal and proportional to r_{ijt} only, as sources taking higher π_j and m_{jt} values can therefore result in more infections attributed to that source.

Attribution estimates of the modified Dutch model seemed to be more precise and consistent between farm and food levels compared to those of our modified Hald model, which seemed to be more sensitive to changes in within-source serotype frequency distribution between farm and food levels. Discrepancies in the estimates between the two models may be explained by the different computational methods they use, as also pointed out elsewhere [15,16,25]. A heterogeneous distribution of the frequently occurring serotypes among the sources is a prerequisite for the Hald model to find the solution with the highest probability of occurrence. Violating such heterogeneous distribution would result in a very diffuse posterior distribution, as the frequency of the so-called "indicator serotypes" on which source attribution relies would be little informative for the model [9]. In our modified Hald models, although converge was adequately achieved, we noted signs of this, as the distributions of the infections attributable to *Gallus gallus* and pigs were rather wide, especially at farm level. In particular, attribution estimates drifted from *Gallus gallus* to pigs at farm level, and away from pigs and turkeys at food level, thereby letting the contribution of *Gallus gallus* to human salmonellosis increase considerably from farm to food. This may be due to the fact that serotypes predominating in *Gallus gallus* and pigs (at least in animals) were also frequently found in other sources (Table 1), but this is also suggestive of an important role of hygiene practices in modifying the within-source serotype distribution along the food chain. Indeed, attribution at the point of production would identify the animal reservoirs of on-farm microbiological contamination prior or during harvesting, whereas attribution at the point of consumption would identify foods as they are prepared and eaten. Thus, because salmonellas may enter the food chain at different points, the contribution of the different sources to human infections is also reasonably expected to vary from one point to another.

High sensitivity of the Hald model to changes in prior information, particularly for the serotype-dependent parameter q_i, has been claimed [15]. We chose to model q_i hierarchically as a random effect with its variation controlled by the hyper-parameter τ like in the modified Hald model [16]. This, together with the use of data split into multiple periods while estimating pooled q_i and a_j parameters over all periods, was expected to improve identifiability and robustness of the model, as reported elsewhere [11,16]. Inherent to the way by which these parameters were estimated is the assumption that the ability of the different serotypes and sources to cause infection in humans are properties of the serotypes and sources themselves and do not change over time. Temporal differences were therefore expected to be explained entirely by the serotype frequency distributions, food consumption patterns and sampling uncertainty.

Attributions made here have some limitations related to data availability in need of further investigations. These were the lack of distinction between broilers and layers/eggs within *Gallus gallus* and the lack of more discriminatory typing data than serotypes only. Furthermore, concerns remain about the heterogeneous distribution of human *Salmonella* infections across the country, as

southern regions are usually more prone to underreporting than northern regions [4,5].

5. CONCLUSIONS

With some differences in consistency and precision of attribution estimates over time periods and sampling points, both our adaptations of the modified Dutch and Hald source attribution models to Italian *Salmonella* data identified pigs as the main source of human salmonellosis in Italy, followed by *Gallus gallus*, whereas the contributions of turkeys and ruminants were estimated to be only minor. This ranking provided us with valuable insights about the relative contribution of these sources to the burden of human salmonellosis in Italy. The increasing importance of pigs and the decreasing one of *Gallus gallus* as sources of human salmonellosis suggest that the applied control measures have been successful in poultry but there is an urgent need to focus attention on pigs. Despite data limitations and uncertainty in the results, our attribution estimates can be considered valid as a first indication of which sources are becoming increasingly important in Italy. These results are expected to be useful for the delineation of future risk management strategies in Italy. Although both our models applied at farm and food levels reached similar conclusions, more detailed data collected at varying levels of the transmission chain may further inform policy makers about the most critical points on which control efforts should be targeted.

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Chapter 5

Practicalities of using non-local or non-recent multilocus sequence typing data for source attribution in space and time of human campylobacteriosis

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Practicalities of using non-local or non-recent multilocus sequence typing data for source attribution in space and time of human campylobacteriosis

ABSTRACT

In this study, 1208 *Campylobacter jejuni* and *C. coli* isolates from humans and 400 isolates from chicken, collected in two separate periods over 12 years in The Netherlands, were typed using multilocus sequence typing (MLST). Statistical evidence was found for a shift of ST frequencies in human isolates over time. The human MLST data were also compared to published data from other countries to determine geographical variation. Because only MLST typed data from chicken, taken from the same time point and spatial location, were available in addition to the human data, MLST datasets for other Campylobacter reservoirs from selected countries were used. The selection was based on the degree of similarity of the human isolates between countries. The main aim of this study was to better understand the consequences of using non-local or non-recent MLST data for attributing domestically acquired human *Campylobacter* infections to specific sources of origin when applying the asymmetric island model for source attribution. In addition, a power-analysis was done to find the minimum number of source isolates needed to perform source attribution using an asymmetric island model. This study showed that using source data from other countries can have a significant biasing effect on the attribution results so it is important to carefully select data if the available local data lack in quality and/or quantity. Methods aimed at reducing this bias were proposed.

1. INTRODUCTION

Campylobacter is the most common cause of bacterial gastroenteritis in the western world [1]. Several source attribution studies have been performed to quantify the relative contributions of different sources of infection to human campylobacteriosis. The results of these studies can be used for identifying those sources of infection that are the most promising targets for *Campylobacter*-reducing intervention efforts, as well as for measuring the impact of such efforts at varying levels of the transmission chain. Chicken has been indicated as the major contributor to the disease burden of human campylobacteriosis in most countries where source attribution studies pertaining to those geographical regions have been performed [2–5]. However, in other countries, ruminants have also been found to be important [6,7]. As new *Campylobacter* sequence types (STs) emerge and the relative occurrence of the established ones change continually, attribution results may vary over time [8]. In addition, the human exposure to *Campylobacter* may vary as well, for example, because of international travel and trade, changes in food consumption patterns and eating habits, either over space or time.

To estimate the proportion of human *Campylobacter* infections attributable to different sources, differences in the relative occurrence of bacterial subtypes in individual sources may be used. The *Campylobacter* spp. subtypes found in human cases and in food and environmental sources are compared to attribute human campylobacteriosis cases to sources. Multilocus Sequence Typing (MLST) [9] has been used as the typing method of choice in most recent studies [3–5,7] as it displays a reasonable level of heterogeneity of *Campylobacter* STs among the different sources. Thus far, most published studies on *Campylobacter* source attribution have been performed in countries in which a relatively large number of local and recent *Campylobacter* spp. strains from animal and environmental sources have been isolated and typed with MLST. Yet, the set up of intensive sampling schemes and the examination of the

collected samples to obtain *Campylobacter* MLST data from multiple sources is costly. As a result, *Campylobacter* MLST data and related source attribution studies are lacking in many countries. It has been noted that, although surprisingly robust [10], the use of non-recent or non-local data in attribution studies may introduce bias into the attribution results for human cases of campylobacteriosis within a country [11]. In addition, the use of a small-sized human or source dataset may result in uncertain and, therefore, less generalizable estimates.

In the Netherlands, *Campylobacter* MLST data have been collected from humans between 2002–2003 and 2010–2011 and from chickens between 2000–2007 and 2010–2011. In this study, we present these data and compare them with other published data from different countries. In addition, we analyze temporal changes in MLST frequencies of such data. Only a small number of local *Campylobacter* MLST reference strains were available from other sources than chicken. A method was proposed to select MLST datasets representing sources other than chicken from international studies to be used for source attribution purposes.

The aim of this study was to better understand the consequences of using non-local or non-recent MLST data for attributing domestically acquired human infections to their putative sources of origin. We investigated how the source attribution model used performs in absence of local or recent data, or when few data are available. Based on these analyses, we give recommendations about which and how many data from other countries should be used for obtaining reliable source attribution estimates if the available local data lack in quality and/or quantity.

2. MATERIALS AND METHODS

2.1. Data

2.1.1. Campylobacter MLST data from the Netherlands

Data of laboratory-confirmed human cases of *Campylobacter jejuni* and *C. coli* infection in the Netherlands were obtained for two different periods. Between April 2002 and April 2003, stool samples were collected from 2858 *C. jejuni* and 257 *C. coli* human cases during a case-control study on risk factors for indigenous campylobacteriosis and salmonellosis, the so-called CaSa study [12]. Of these, 948 *C. jejuni* and 66 *C. coli* isolates were subsequently successfully typed with MLST [9] to be used for source attribution and source-specific risk characterization [2]. Of these, 743 cases (699 *C. jejuni* and 44 *C. coli*) were domestic cases, as the other cases had a recent history of foreign travel.

Isolates from more recent domestic human cases of campylobacteriosis routinely identified by the Dutch Regional Public Health Laboratories through passive surveillance were obtained between June 2010 and June 2011. In total, another 423 *C. jejuni* and 42 *C. coli* strains were typed using MLST.

In addition, 218 *Campylobacter* isolates from fresh retail chicken meat of Dutch origin, sampled between 2000 and 2007, were obtained. More recent chicken isolates of Dutch origin were obtained between 2010 and 2011. These consisted of 158 isolates from retail chicken meat and 24 isolates from layer hens, pooled together assuming that layers and chickens are a single reservoir (*Gallus gallus*). Isolates from other *Campylobacter* sources in the Netherlands were obtained between 2000 and 2006 (cattle, n = 9; pigs, n = 13; environmental water, n = 106). These isolates were also typed using MLST [9].

In this study, cases with a recent history of foreign travel were excluded and *C. jejuni*/*coli* data were given at the species level for humans but not for chicken isolates.

2.1.2. Campylobacter MLST data from international studies

A literature review was conducted to identify published studies that provide MLST data for *Campylobacter* isolates from human cases and from various sources in countries other than the Netherlands. It was required that the data in such studies were representative of the natural strain diversity and relative frequencies therein, in those countries. Therefore, studies presenting isolates which are subject to any form of selection (e.g. reporting of novel strains only) were excluded. Isolate collections used in this study are shown in Table 1.

2.2. Comparison of datasets

2.2.1. Analysis of diversity

The distributions of ST frequencies in different datasets were compared visually by stacking the frequency bars of the most common STs found in the different studies next to each other. In addition, the frequency distributions of the most common STs and clonal complexes (CCs) in different datasets were compared with one another to allow for genetically close relationships between STs within the same CC to be evidenced. Approximate confidence intervals (CIs) for the ST or CC frequencies were calculated using bootstrapping [13].

The proportional similarity index (PSI, or Czekanowski index) [14] was used to measure the similarity of frequencies of STs between the different datasets. The PSI is expressed as $PSI = 1 - 0.5 \sum_k |P_k - Q_k|$, where $|P_k - Q_k|$ is the absolute value of the difference in the relative frequency of MLST genotype k in dataset P compared to its frequency in dataset Q. The values of PSI range from 0 to 1, with 0 indicating that both distributions have no types in common and 1 that both distributions are completely equal. CIs for the PSI were also calculated using bootstrapping [13].

2.2.2. Principal component analysis

In addition to the numerical similarities measured by the PSI between datasets, a principal component analysis (PCA) [15] provides additional insights towards which STs are the main contributors to the differences observed between the different datasets. Briefly, the original coordinate system, in which each axis represents the relative frequency of one ST in the datasets, is linearly transformed by PCA. In the transformed coordinate system, most variability is explained by the first coordinate (the first principal component), the second largest variability is explained by the second coordinate, etc. The proportion of the variability that is explained by the *n*th coordinate equals the fraction of the *n*th eigenvalue out of the summed total of all eigenvalues of the transformation matrix. A plot of the transformed axes shows which STs are most relevant for explaining the differences between the datasets. If the first dimensions of the transformed system explain the majority of variability, only these need to be plotted.

2.3. Source attribution

2.3.1. Asymmetric Island model

The large effective population size of *Campylobacter* causes frequent mutation despite a relatively low mutation rate per allele [16]. Also, *Campylobacter* recombines [17] and migrates from one host to another [18]. With the Asymmetric Island (AI) model [5], the parameters describing these genetic changes within, and drift between, the source populations are inferred using Bayesian inversion. Subsequently, they are used for comparing one group of isolates (the attributable population) to other groups (the source populations). For each case, the AI model estimates a relative assignment posterior probability (*Pr*) to originate from each source. The proportion of human infections attributed to a given source is calculated as the average *Pr* over all cases. The AI model has been used for source attribution in a number of published studies [5,19,20] and has been reported to provide results with a relatively high level of confidence [19].

2.3.2. Baseline attribution analysis

In the baseline attribution analysis, the attributable population consisted of the 1208 non-travel related Dutch human cases in 2002–2003 and 2010–2011. The source populations were defined by the available MLST source data from the Netherlands supplemented with MLST source data from a selection of other published studies. Supplementary source data were used from countries where the human isolates were most similar to Dutch human isolates, as indicated by the PSI. Isolates that were used in the baseline attribution analysis are printed in bold in Table 1. The augmented dataset is composed in such a way that there were 168 isolates for cattle, 160 for sheep, 133 for pig and 289 for the environment. Chicken data from countries other than the Netherlands were not used because sufficient Dutch data were available for this source.

2.3.3. Advanced attribution analyses

Typically, the available *Campylobacter* MLST data for source attribution are imperfect [2]. Source data are in fact scarcer than human data in many countries because these are not collected routinely. To verify the impact of imperfect source data, the following scenarios were tested:

- Source attribution with non-local source data.
- Source attribution with limited source data.

The impact of using non-local source data was assessed by performing the following source attribution analyses of Dutch human cases and comparing their results to the baseline attribution results. First, chicken data from countries relatively close to the Netherlands (UK, here Scotland and England, and Switzerland) were used instead of the domestic chicken data. The effect of using chicken data from countries further away from the Netherlands (New Zealand, Finland and the US) was also investigated. Ultimately, domestic chicken and chicken from Scotland, England and Switzerland were considered as distinct sources in the attribution analysis.

The impact of using non-local source data was further studied by letting non-local chicken isolates be the attributable population, and attributing these using the source populations as defined in the baseline attribution analyses (bold numbers in Table 1). This type of analysis is called self-attribution, and can also be used to test the statistical power of the attribution model [19]. In this case, a high similarity between the non-local and local chicken isolates and a high statistical power of the model should result in a self-attributed proportion of the chicken isolates that is close to 1.

Self-attribution was also used to study the impact of using source data with a limited sample size. Of the 400 chicken isolates used in the baseline attribution, 250 were randomly selected to be the attributable population. Experiments indicated that the effect of modifying this initial split of the chicken data on the attribution results was negligible; thus, only one random split was considered in the following experiments. The remaining 150 chicken isolates and 150 randomly selected isolates from the remaining source populations (as defined in the baseline attribution model) were the reduced source populations. Subsequently, self-attribution was carried out. Self-attribution of the same 250 chicken isolates was also done with random subsets of 100, 75 and 50 isolates from each source population to explore the effects of using smaller-sized source datasets. To account for variability in the attribution results caused by the random subset selection, self-attribution was done 10 times for every subset of the source population, while keeping the attributable population of 250 chicken isolates constant.

Finally, a source attribution analysis based on the minimum possible non-recent and non-local data was performed. This was made by letting the human cases in 2002-2003 be the attributable population and using the corresponding Dutch source data (NL1 dataset in Table 1) supplemented with only the most similar non-Dutch source data (SC dataset in Table 1).

Table 1: Number of isolates in published human (h) and source (s) datasets and (last column) bootstrapped similarities of the human data with the human data in the NL1 dataset

Set	Ref.[a]	Country (region)	Year	Human	Chicken	Cattle	Sheep	Pig	Environnent	PSI (95%CI)[b] of human data to human data of NL1
NL1	[2]	Netherlands	h: 2002–2003; s: 2000–2007	**743**	**218**	**9**	0	**13**	**106**	
NL2	Data	Netherlands	2010–2011	**465**	**182**	0	0	0	0	0.41 (0.36–0.44)
SC	[4]	Scotland (Grampians)	2005–2006	278	239	**90**	**88**	**15**	**133**	0.40 (0.36–0.43)
CH2	[29]	Switzerland	2009	383	0	0	0	0	0	0.35 (0.31–0.38)
CH	[24]	Switzerland	h: 1993–2003; s: 2001–2002	76	77	**23**	0	**100**	0	0.31 (0.24–0.38)
CH3	[22]	Switzerland	2008	136	0	0	0	0	0	0.31 (0.27–0.35)
EN	[9]	England	h: 1977–2001; s: 1983–1999	355	73	**46**	**72**	**5**	**50**	0.30 (0.26–0.33)
NL3	[9]	Netherlands	h: 1996–1998; s: 1990–1999	76	53	3	0	0	0	0.29 (0.22–0.36)
NZ1	[3]	New Zealand (Manawatu)	2005–2008	502	331	99	140	0	104	0.26 (0.22–0.29)
SP	[6]	Spain	h: 2003–2009; s: 2003–2006	71	36	80	44	0	0	0.22 (0.15–0.27)
FI	[30]	Finland	h: 1996, 2002–2003; s: 2003	305	36	20	0	0	0	0.21 (0.18–0.25)
NZ2	[31]	New Zealand	2006	112	0	0	0	0	0	0.20 (0.15–0.26)
CUR	[26]	Curacao	1999–2000	205	0	0	0	0	0	0.19 (0.15–0.23)
AU	[27]	Australia (New South Wales)	1999–2001	153	0	0	0	0	0	0.18 (0.14–0.23)
US	[32]	USA (Michigan)	2003	17	13	0	0	0	0	0.14 (0.06–0.21)

a. The datasets are ordered in decreasing similarity of the human isolates with the NL1 human data. Numbers of isolates that are written in bold were used in the baseline source attribution analysis.

b. Proportional Similarity Index, with 95% confidence intervals.

3. RESULTS

3.1. Temporal variation

A large variety of STs was found in the Dutch human and chicken data. In Figure 1, the contributions of those CCs including STs that were found in the human data of 2002–2003 in proportions over 1% (accounting for 83% of all isolates) are represented together with the contributions of the same STs within these CCs for chicken data of 2000–2007 and for human and chicken data of 2010–2011; these CCs accounted for 65%, 67% and 52% of all isolates, respectively.

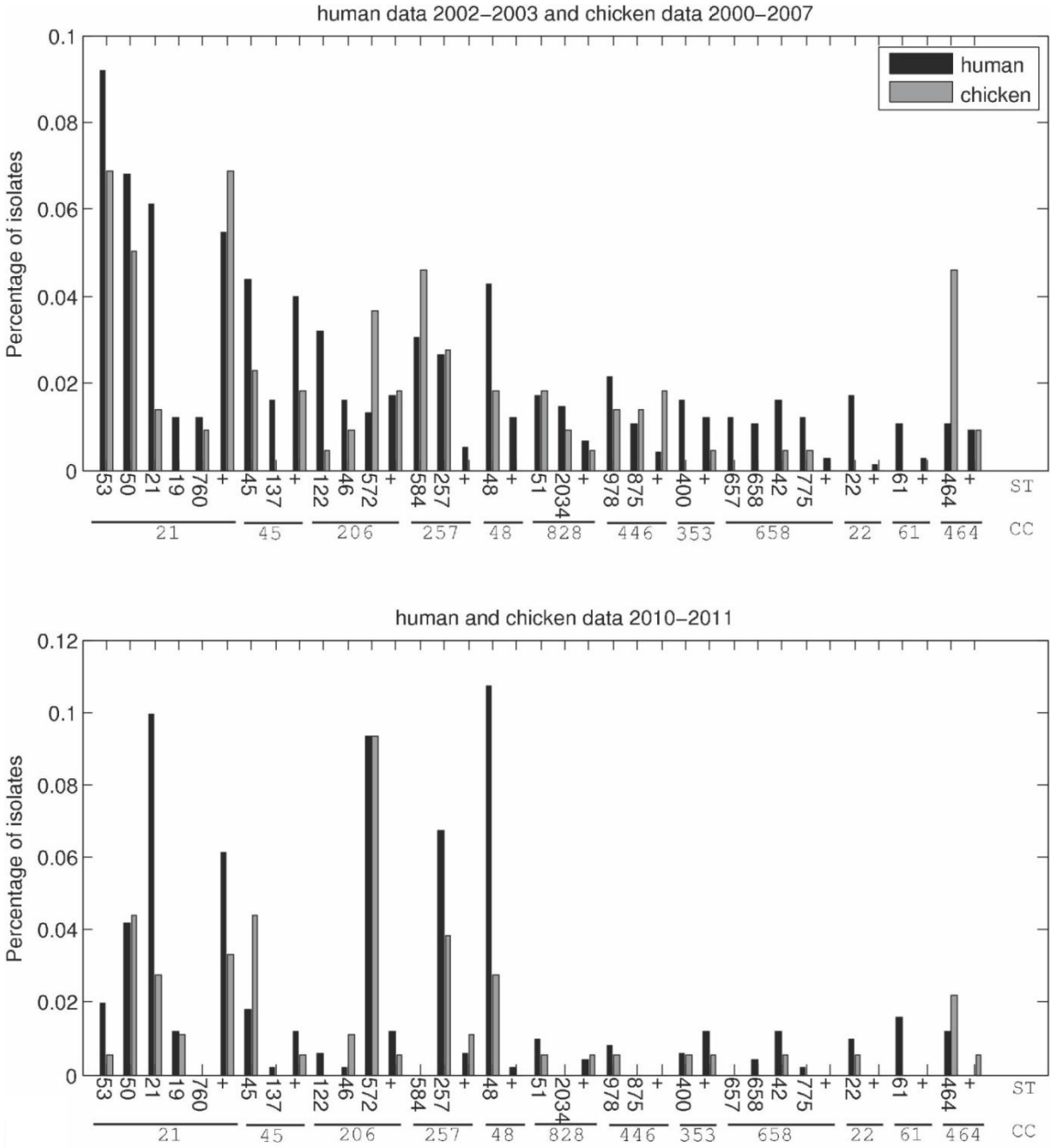

Figure 1. Most common STs in human and chicken isolates in The Netherlands in two time periods. Only the contributions of those CCs including STs that were found in the human data of 2002–2003 in proportions over 0.01 are represented. The contributions of less frequent STs within these CCs are summed and presented by the "+" symbol; the contributions of other CCs are omitted. For the human data of 2002–2003 the presented CCs make up for 83% of all isolates. For the chicken data of 2000–2007 and the human and chicken data of 2010–2011, these CCs make up for 65%, 67% and 52% of all data, respectively.

Among the 743 human isolates from 2002–2003, 161 different STs were observed. The five most frequent STs were ST53 (9.2%), ST50 (6.8%), ST21 (6.1%), ST45 (4.4%) and ST48 (4.3%). Among the 218 chicken isolates from 2000–2007, 87 different STs were found, the five most common STs being ST2483 (7.8%), ST53 (6.9%), ST50 (5.0%), ST584

(4.6%) and ST464 (4.6%). In the 465 human isolates from 2010–2011, 129 different STs were observed. The five most frequent STs were ST48 (10.7%), ST21 (9.9%), ST572 (9.3%), ST257 (6.7%) and ST50 (4.2%). Among the 182 chicken isolates from 2010-2011, 82 different STs were found, the five most common STs being ST2274 (11.5%), ST572 (9.3%), ST50 (4.4%), ST45 and ST257 (3.8%).

The PSI was used as a tool to quantify the (dis)similarity between recent and non-recent isolates. Figure 2 indicates that the STs isolated from chicken and human cases are increasingly dissimilar as the period between which the samples were taken increases. The linear decrease is borderline significant with a mean slope of –0.011 (95% CI: –0.018 to –0.003). PSI was also calculated for chicken data between 2000–2004 and 2005–2007 (PSI = 0.24, 95% CI: 0.11–0.38), between 2000–2004 and 2010–2011 (0.20, 0.07–0.33), and between 2005–2007 and 2010–2011 (0.34, 0.22–0.46). Although the 95% CIs overlap one another, a trend is notable towards dissimilarity of chicken data as the period between which the samples were taken increases.

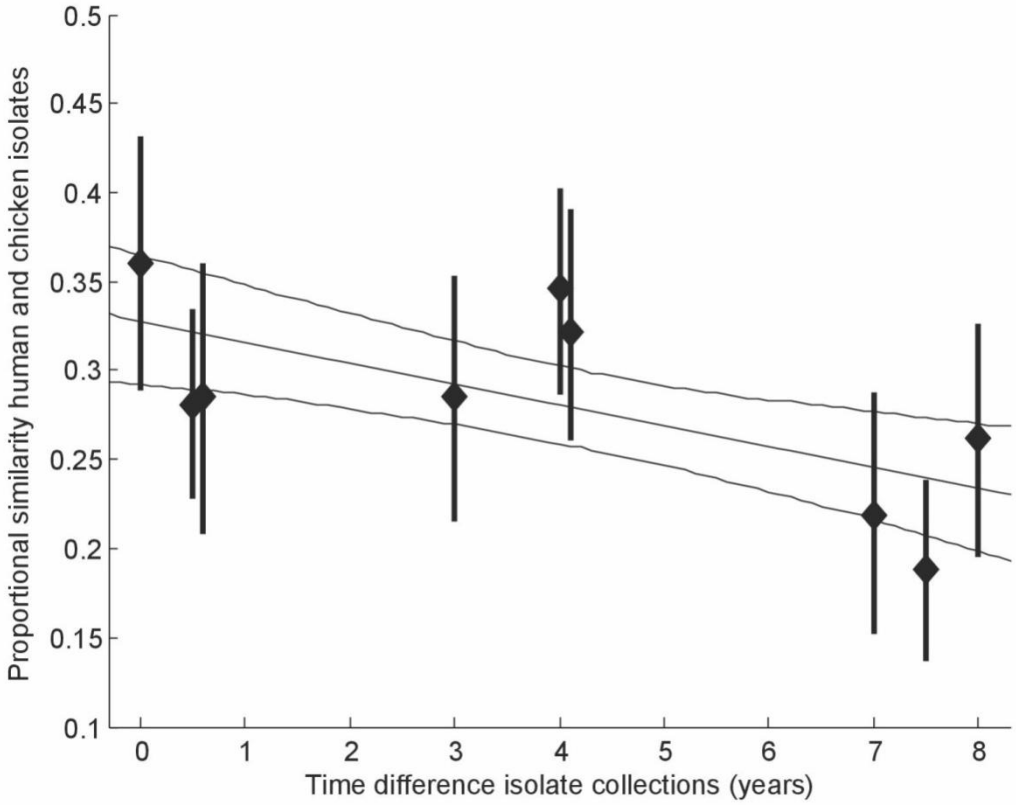

Figure 2. Similarity of STs in chicken and in human isolates from samples collected in different years. The x-axis gives the absolute difference between years in which the isolates from human cases and chicken were obtained. To enhance the size of the sample subsets, chicken isolates collected between 2000 and 2004 were aggregated and assigned to be collected in 2002, those collected between 2005 and 2007 were assigned to be collected in 2006, and those collected in 2010–2011 were assigned to be collected in 2010. Human isolates were arranged in three groups: 2002, 2003, and 2010–2011. The y-axis represents the PSI between those isolates collections.

3.2. Geographical variation

The frequency distributions of the most common CCs in human datasets published in the international literature are shown in Figure 3. The most commonly found CC in England is CC21, followed by CC45, CC48 and CC257. For the Dutch human data, these were also important CCs in 2002-2003 as well as in 2010–2011. CC21 was less common in human cases in other countries, in particular in Australia and in the US. CC48 was remarkably prominent in New Zealand. This is mainly due to CC48 member ST474, which accounted for 30% and 29% of all human cases in the two New Zealand studies, respectively. ST45, the founder strain of CC45 was by far the most common ST in Finland, accounting for 28% of the human cases. CC354 member ST528, which is frequently reported in New South Wales, Australia, was not reported in the other studies.

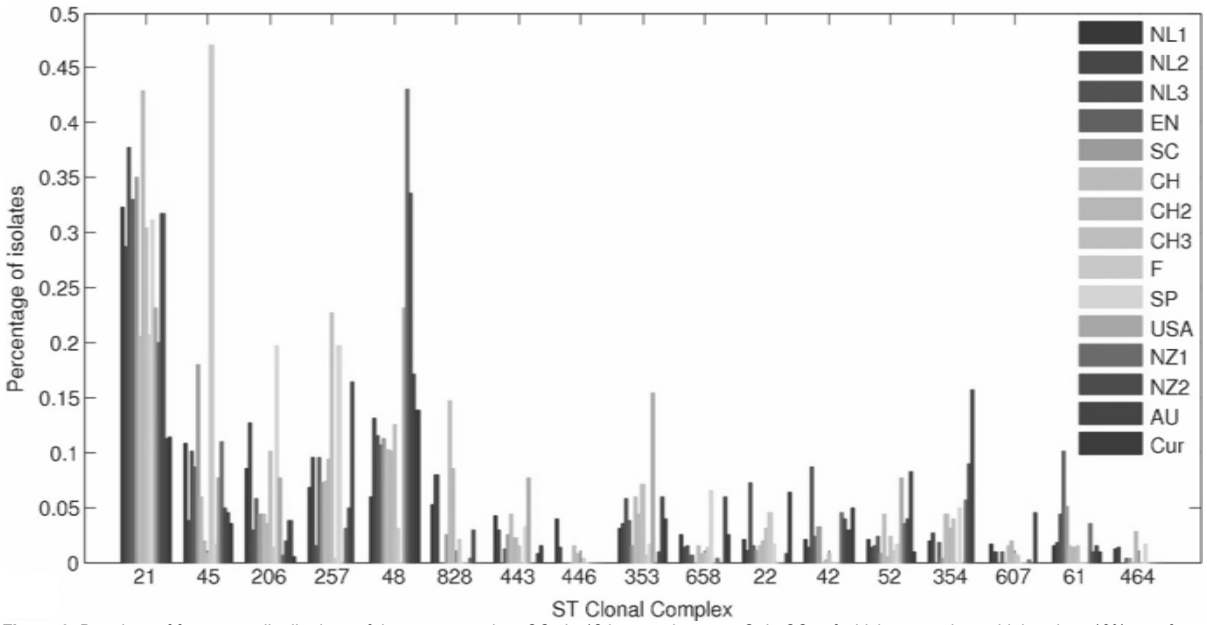

Figure 3. Bar chart of frequency distributions of the most prevalent CCs in 12 human datasets. Only CCs of which a prevalence higher than 10% was found are plotted.

The analysis of similarity of the Dutch human data from 2002-2003 with the human data from other datasets shows that they are most similar to Dutch human data from 2010–2011, followed by human data from Scotland, England and Switzerland (Table 1). In general, the Dutch human data were less similar to data from Finland, Spain and the considered non-European countries. This was expected because of the differences in geographical distance. All international datasets were significantly different from the Dutch human data from 2002–2003, as can be seen by the fact that the similarity 95% CI within these Dutch data does not overlap any other similarity confidence interval (Table 1).

In Figure 4, the first three dimensions of the PCA transformed system of ST frequency vectors are plotted. ST474 sets the datasets from New Zealand apart from other datasets and ST21 sets datasets from Switzerland slightly apart from other datasets. The Finnish dataset is set apart from other datasets by a high prevalence of ST45 and the dataset from Curacao is set somewhat apart from other datasets due to a high prevalence of ST508. Evaluation of the eigenvalues of the transformation matrix obtained in the PCA showed that the first three dimensions of the transformed coordinate system explain about 73% of the variability of ST frequencies between the datasets.

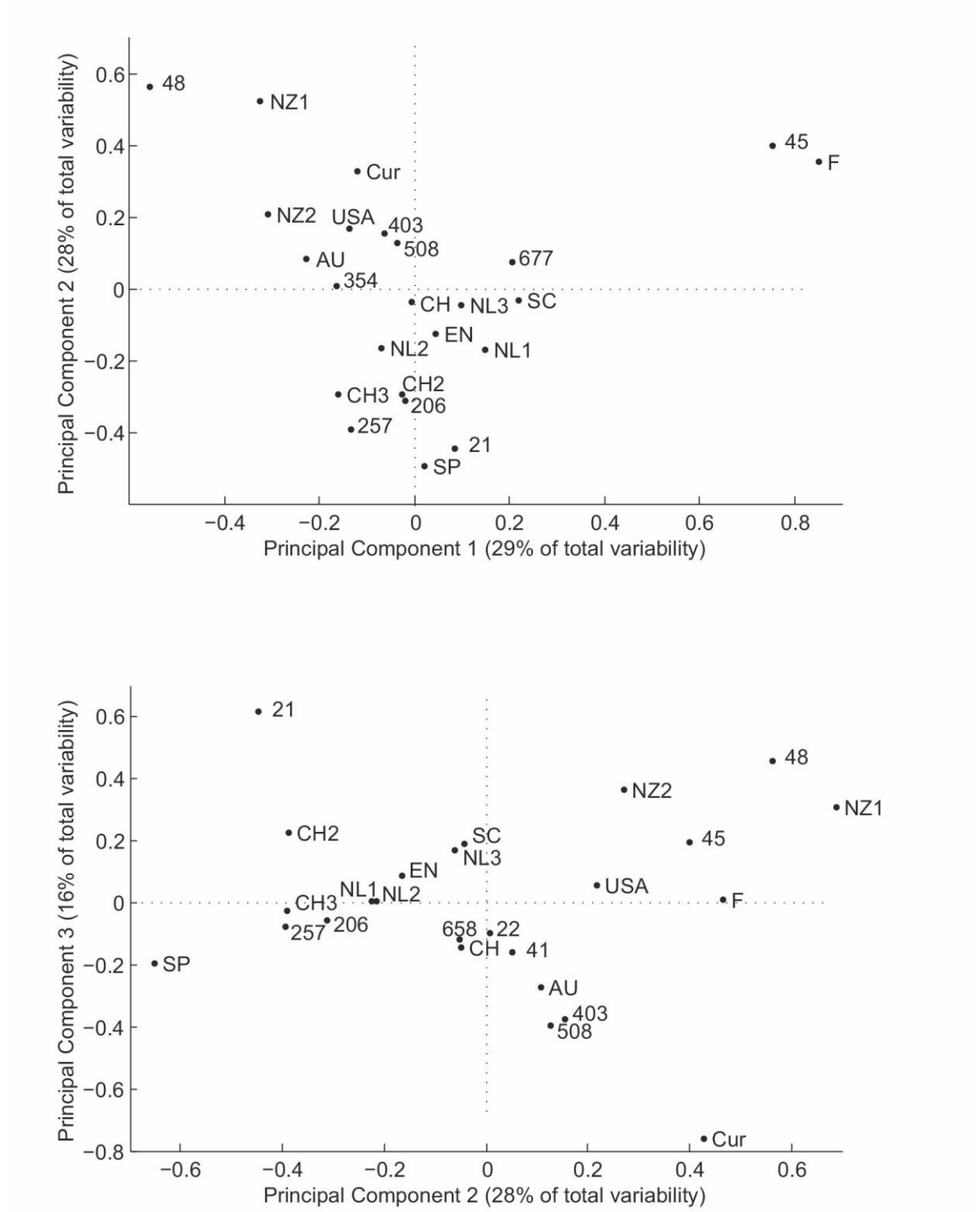

Figure 4. PCA transformed vectors of CC frequencies in 12 human datasets. The first, second and third PCA transformed dimensions explain together 73% of the total variability in the data. Weighted sums of the CC frequency distributions of the human isolates in the different datasets reported in Table 1 are plotted in the first two (upper graph) and in the second two (lower graph) dimensions of the PCA transformed space.

3.3. Attribution analyses

In the baseline attribution analysis (Figure 5A), of all 1208 human cases of campylobacteriosis, 68% (95% CI: 61–74%) was attributed to chicken, 24% (18–31%) to cattle, and 6% (2–10%) to the environment, while the contributions of sheep and pig were only minor (2% together). If the Dutch chicken data were replaced by chicken data from Scotland, England and Switzerland (Figure 5B), then the importance of chicken for human disease decreased to 45% (37–52%), whereas the contributions of non-chicken sources increased. Replacement of the Dutch chicken data by chicken data from New Zealand,

Finland and the US (Figure 5C) greatly reduced the inferred role of chicken for human disease (20%, 14–25%), leading to cattle being the most important source (45%, 36–55%), followed by the environment (32%, 23–40%). When data from domestic chicken and data from Scottish, English and Swiss chicken were considered as separate sources in the model (Figure 5D), then it is evident that there is much more overlap of MLST genotypes between the domestic chicken and Dutch human isolates (63%, 55–70%) rather than non-Dutch chicken (17% together).

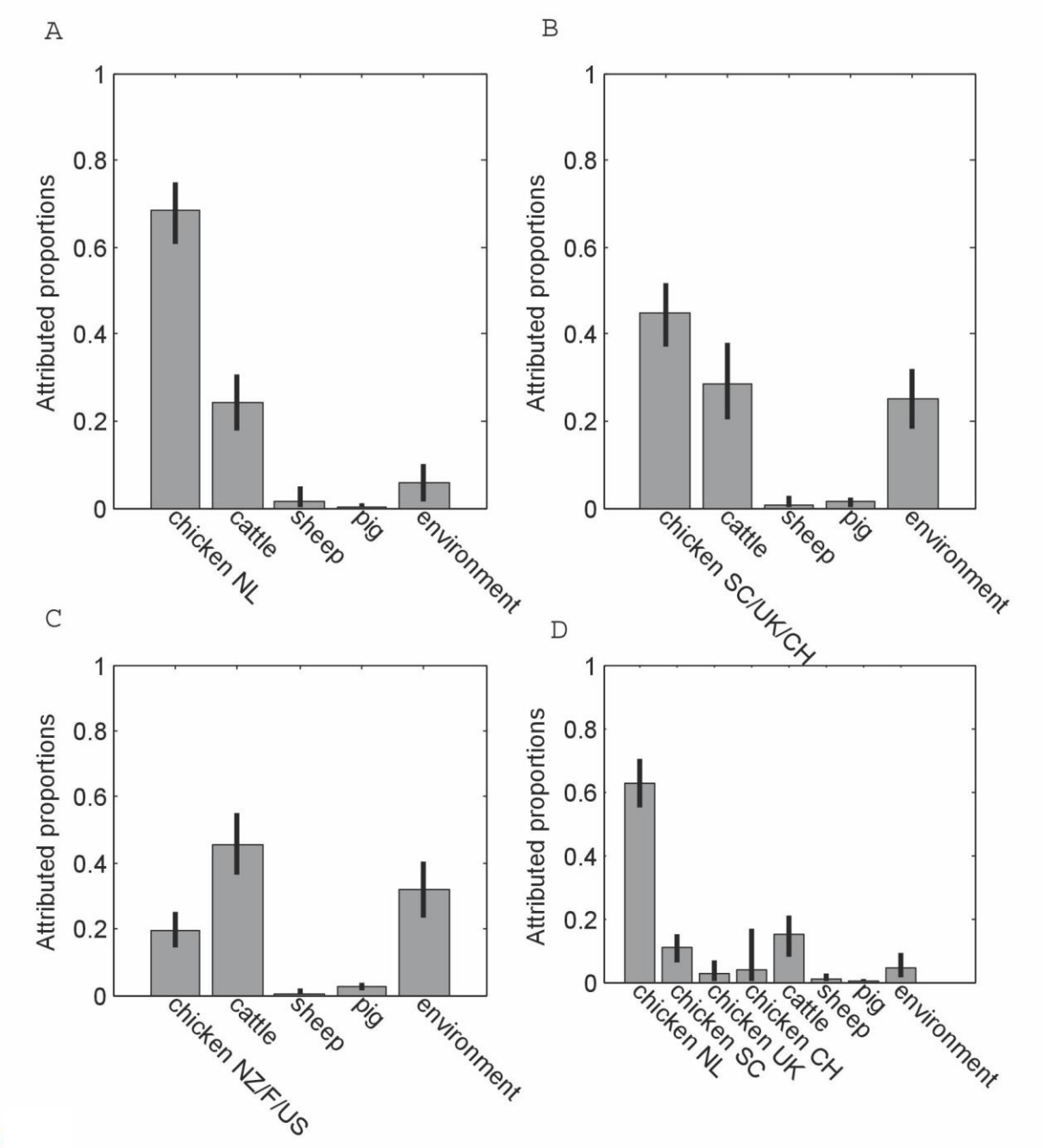

Figure 5. Overall mean probability (%) and 95% confidence interval for human C. jejuni and C. coli infections to originate from chicken, cattle, pig, sheep, and the environment. A. Baseline attribution results (see main text); B. Attribution results with Dutch chicken isolates replaced by chicken isolates from Scotland, the UK and Switzerland; C. Attribution results with Dutch chicken isolates replaced by chicken isolates from New Zealand, Finland and USA; D. Attribution results with Dutch, Scottish, English and Swiss chicken isolates as separate Campylobacter reservoirs.

If the self-attribution analysis were done with domestic chicken as the attributable population and the source populations the same as in the baseline attribution analysis, then 89% (81–95%) of these isolates were attributed to the right source. If chicken isolates from Scotland, England and Switzerland were assigned as the attributable population, then the percentage of correct self attribution was 62% (47–75%). Similarly, if chicken isolates

from New Zealand, Finland and the US were assigned as the attributable population then 62% (49–73%) of these isolates were attributed to the right source.

Figure 6 shows the impact of using limited source data. It is seen that the variability over the mean attributed proportions (caused by randomly generating reduced datasets) increases for smaller subsets of the original source data. This is evident as the random effects increase for these smaller subsets. Also the statistical power of the AI model decreases if fewer data are available, which leads to a larger uncertainty. This implies that the confidence of the attribution results decreases as fewer data are available. The statistical power of the attribution model was fairly robust for smaller-sized source datasets until a minimum number of 100 isolates per source. If fewer than 100 isolates are available per source then the statistical power of the attribution model decreased substantially. For an average 2.5% confidence over 50% of correct source attribution, it is advisable that more than 25 isolates per source are used.

In the attribution analysis based on the minimum possible non-recent and non-local data (where the word "minimum" here refers to the supplementary non-Dutch source data used in the model and not to the sample size), 63% (95% CI: 56–69%) of the 743 human cases of 2002-2003 was attributed to chicken, 25% (19–32%) to cattle, and 11% (6–15%) to the environment, while the contributions of sheep and pig were again minimal (1% together).

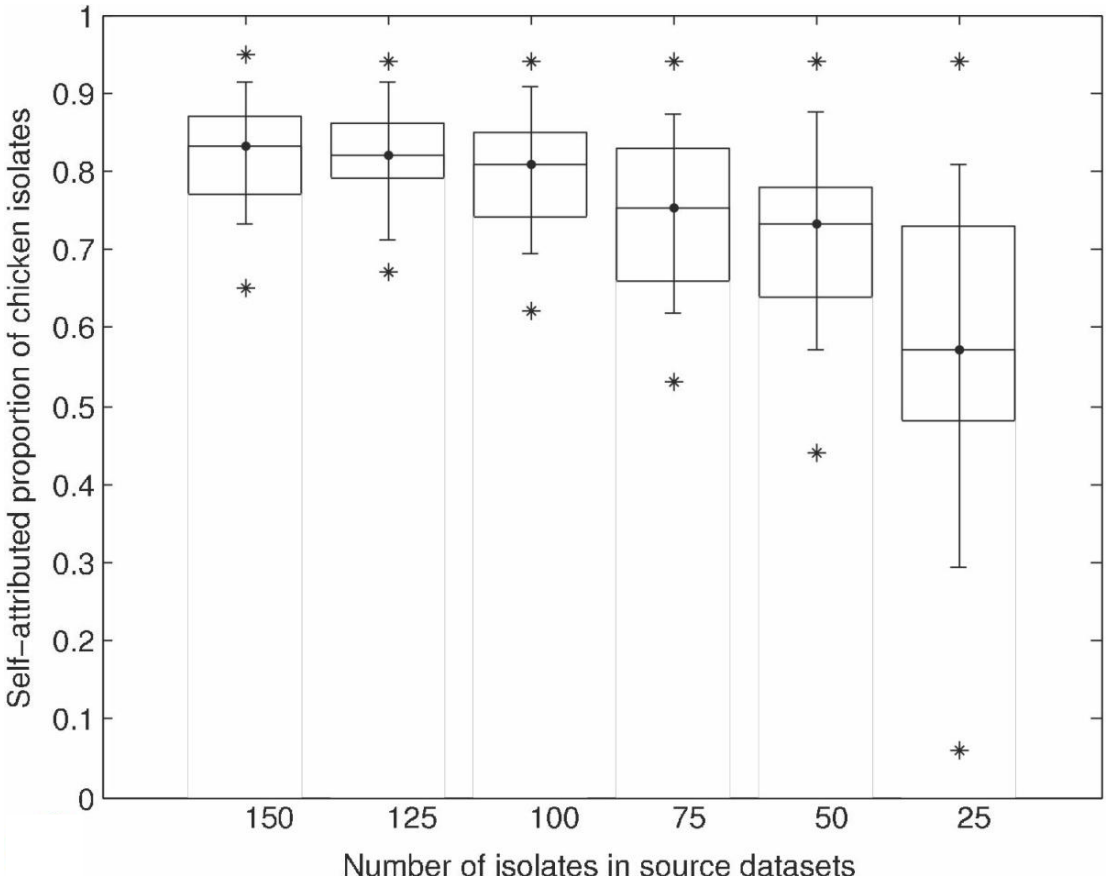

Figure 6. Statistics of the self-attributed proportions of 250 chicken isolates for reduced source datasets of size n (on x-axis). Every reduced dataset is generated from the original dataset by randomly removing isolates from an original set of 150. The boxes indicate variability in the mean attributed proportions over the 10 different reduced datasets per model and per reduction factor. Indicated are the minimal, maximal and average means. The whiskers indicate the average 2.5% and 97.5% confidence limits over the different reduced datasets. The star-symbols represent the minimum 2.5% limit and the maximum 97.5% limit.

4. DISCUSSION

We presented the results of a study in which *Campylobacter* isolates from Dutch human patients (*n* = 1208) and Dutch chicken (*n* = 400) collected between 2002–2003 and 2010–2011 were typed using MLST. The large size of this dataset provided the opportunity to perform a multitude of analyses aimed at defining the effect of time and geographical location on the diversity of the *Campylobacter* population. Other reservoirs for

Campylobacter were less well sampled in the Netherlands. Therefore, non-local source data were used to supplement the Dutch ones in order to attribute the human infections to the different sources. A practical method was also proposed to select such supplementary data with the aim of minimizing potential biases of the attribution estimates. This method is based on the assumption that if the human data between different countries and time periods resemble one another (as revealed by PSI and PCA), then also will their respective source data, which may therefore be borrowed interchangeably for the purposes of source attribution. Inherent to this way of choosing the source data is the assumption that the consumption patterns and exposure pathways from sources to humans are similar in the Netherlands and in the countries/time periods from which the supplementary source data were collected, and that diversity between the human datasets can only be explained by intrinsic differences in the genotype distribution and by sampling uncertainty.

The attribution analyses showed that chicken was the most important source of human campylobacteriosis in the Netherlands, accounting for 61–74% of the human cases in the baseline model where the two human datasets for 2002–2003 and 2010–2011 were pooled based on their high similarity and the fact that the corresponding source data covered on average the whole time period. This is in line with findings from previous source attribution studies conducted in several other countries [2,3,7,21,22]. Nevertheless, our analyses suggest that the high proportion of human cases attributed to chicken and the smaller proportions of cases attributed to non-chicken sources (which are less intensively sampled in the Netherlands) may depend on the origin of the source data included in the model. When domestic chicken data were replaced by chicken data from countries showing the closest possible human MLST profiles to those of the Netherlands, i.e. Scotland, England and Switzerland, the ranking of sources remained the same as that of the baseline model but the contribution of chicken to human cases decreased considerably. This was more evident and the ranking of sources was even reversed when domestic chicken data were replaced by chicken data from countries with human MLST data less similar to those of the Netherlands, i.e. New Zealand, Finland and the US. Moreover, when Dutch, Scottish, English and Swiss chicken data were included as separate sources, it became apparent that domestic chicken is much more important than foreign chicken in accounting for domestic human cases. Together these findings suggest that the further in region and time one takes the source data, the more their MLST profiles will differ, and the smaller will be the estimated proportions of human cases attributable to those sources that were sampled less close in time and space to the human cases.

ST50 is shared as a common ST among the human and chicken isolates collected in the periods 2002–2003 and 2010–2011, and results from the AI model showed that human cases with ST50 had a 90% probability of having been infected by chicken or by strains with chicken origin. This ST belongs to CC21, which is reported to have a relatively wide distribution across many host species but slightly more dominant in ruminants [23]. Other STs belonging to this complex are ST21 and ST53. ST21 was more common in human cases than in chicken in both periods. Results of the AI model showed that human cases with ST21 were slightly more likely to have been infected by ruminants (*Pr* = 0.51) than by chicken (*Pr* = 0.43). The decline of ST53 in samples from chicken, being the most frequent ST in samples from 2000–2007 but a minor ST in samples from 2010–2011, coincided with a decline of this ST in the human samples as well. A similar decline was seen for ST584 in the chicken and in the human samples. This may indicate the importance of chicken as the source for campylobacteriosis caused by these STs. Results of the AI model confirmed that the probability that these STs originated from chicken was 0.84 and 0.97 for ST53 and ST 584, respectively. In contrast, ST2274 was increasingly common in chicken samples, which coincides with an increase of this ST in the human samples. Results from the AI model showed that human cases with ST2274 were most likely to have been infected by chicken (*Pr* = 0.97). The predominant STs in human data in the Netherlands in 2002–2003 were ST53 and ST50, both belonging to CC21. Also in other studies [7,20,21,24], these strains were reported to be common in human patients.

By comparing the human datasets from several countries to the Dutch human data, it was concluded that the importance of the differences in ST frequencies is correlated with the geographical distance between the countries, with the data from nearby European

countries being generally more similar than data from more distant countries with respect to the Netherlands, such as New Zealand, Australia and the US. PCA was proposed as a method to show in a visually appealing way the difference in occurrence of STs in different studies. The transformed vector representing the Dutch human data is relatively close to the origins of these PCA plots. This indicates that the 2002–2003 Dutch human dataset does not contain one or more CCs in markedly different frequencies than the average frequency distribution over all datasets that were considered. This may be caused by the ease of traveling and trade within the European Union, which leads to a larger exposure to *Campylobacter* from reservoirs present in European countries. However, limited exposure to this international diversity of *Campylobacter* strains may occur in people living in countries where there is a less open national market such as New Zealand or Australia, or where less international importation of meat products, including poultry meat, takes place, such as Spain or Finland. Indeed, approximately 8% and 11% of the total amount of meats available for consumption in 2000–2009 in Spain and Finland were imported, respectively, and these figures are considerably lower than those for the Netherlands (~45%), the UK (~30%), and Switzerland (~16%) [25]. Human isolates from Curacao were taken from Guillain-Barré cases [26], which is a particular subset of campylobacteriosis cases. These may be reasons that studies in these countries show different frequencies of certain CCs compared to the averaged frequencies over all studies, which may be seen by the larger distance from the origins in the PCA plots. Also the CCs that set the studies from these countries apart from other studies are shown in the PCA plots. Indeed, CC48, in particular the CC48 member ST474, is reported in New Zealand more frequently than in other countries [20], ST528, belonging to CC354, is more frequently reported in New South Wales, Australia [27], and the CC45 member ST45 is more frequently reported in Finland [7].

The PCA shows only those CCs which explain the largest variation between the different datasets. Yet, in many studies the same STs (e.g. ST21, ST22, ST48 and ST 257) turn up as the predominant strains. This provides evidence to the suggestion made by Mickan et al [27] that some STs have a global distribution, while others are restricted in their distribution to a more local environment, however the "local STs" may be more associated with countries with less international travel and trade [28].

The results of our study show that it is recommended to have over 100 isolates per food source to perform source attribution using the AI model in order to have satisfactory statistical power. More detailed research questions with respect to attribution estimates might ask for more precision, hence a larger strain set. If this amount of data is not available for each potential source when using only recent and domestic data, then the investigator may be forced to use non-recent or non-local data. We have shown that the MLST data supply for *Campylobacter* within a food source is subject to dynamic changes in time and over geographical location; thus, in principle, this introduces temporal and geographical bias into the study.

As the AI model is based on a population genetics approach, source data collated from studies that show large variations between isolates obtained from the same sources but from different datasets may distort the gene frequencies upon which source attribution relies [5]. Sample size may impact on such variation by letting certain sources to exhibit relatively more unique (with respect to humans and the other sources) genotypes than others; thus, more intense sampling of small-sized sources is generally desirable, as oversampling certain sources relative to the others does not seem to affect the point estimates but only their accuracy [5]. Indeed, the source dataset becomes denser and better defined in terms of representative genotypes by increasing the number of samples. Therefore, notwithstanding the distortion of gene frequencies due to the pooling of source datasets from different studies, this may become less important with increasing sample size.

In conclusion, we have shown that, even on a small time-scale, MLST data within two sources become increasingly dissimilar as the time between different datasets are collected increases so that the AI model may underestimate the importance of a source whose data are not collected contemporaneously with the human cases to be attributed. Temporal bias can be minimized by choosing the most recent data that are available for a source. In addition, the AI model may underestimate the importance of sources from which non-local source data were used. A

coarse rule is that this bias increases with the geographical distance between the countries in which the attribution is performed and from which source data are used. Nevertheless, our results show that geographical distance is not the only factor, and it may act together with factors related to travel and trade between countries. It also has been found that association of genotypes to a particular host is reported to be stronger than their association to a geographical location [10]. Our results show that, although this may make the consequences of geographically biased data less severe, it does not fully compensate for them (Figure 5). In general, the extent to which this bias is a matter of concern depends on how detailed (in time and region) is the research question to be addressed. A method based on the comparison of human isolates from different studies using PSI and PCA was proposed to select non-recent and non-local MLST datasets for the purposes of source attribution while minimizing potential biases.

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Chapter 6

Risk factors for campylobacteriosis of chicken, ruminant, and environmental origin: a combined case-control and source attribution analysis

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Risk factors for campylobacteriosis of chicken, ruminant, and environmental origin: a combined case-control and source attribution analysis

ABSTRACT

Background: Campylobacteriosis contributes strongly to the disease burden of food-borne pathogens. Case-control studies are limited in attributing human infections to the different reservoirs because they can only trace back to the points of exposure, which may not point to the original reservoirs because of cross-contamination. Human Campylobacter infections can be attributed to specific reservoirs by estimating the extent of subtype sharing between strains from humans and reservoirs using multilocus sequence typing (MLST).

Methodology/Principal Findings: We investigated risk factors for human campylobacteriosis caused by Campylobacter strains attributed to different reservoirs. Sequence types (STs) were determined for 696 C. jejuni and 41 C. coli strains from endemic human cases included in a case-control study. The asymmetric island model, a population genetics approach for modeling Campylobacter evolution and transmission, attributed these cases to four putative animal reservoirs (chicken, cattle, sheep, pig) and to the environment (water, sand, wild birds) considered as a proxy for other unidentified reservoirs. Most cases were attributed to chicken (66%) and cattle (21%), identified as the main reservoirs in The Netherlands. Consuming chicken was a risk factor for campylobacteriosis caused by chicken-associated STs, whereas consuming beef and pork were protective. Risk factors for campylobacteriosis caused by ruminant-associated STs were contact with animals, barbecuing in non-urban areas, consumption of tripe, and never/seldom chicken consumption. Consuming game and swimming in a domestic swimming pool during springtime were risk factors for campylobacteriosis caused by environment-associated STs. Infections with chicken- and ruminant-associated STs were only partially explained by food-borne transmission; direct contact and environmental pathways were also important.

Conclusion/Significance: This is the first case-control study in which risk factors for campylobacteriosis are investigated in relation to the attributed reservoirs based on MLST profiles. Combining epidemiological and source attribution data improved campylobacteriosis risk factor identification and characterization, generated hypotheses, and showed that genotype-based source attribution is epidemiologically sensible.

1. INTRODUCTION

Virtually all people in The Netherlands (~16 million population) possess serological evidence of multiple exposures to *Campylobacter* spp. during the course of their lives, although most infections pass with no, or mild, symptoms [1]. With an estimated 90,000 symptomatic infections occurring annually, campylobacteriosis is the most frequent cause of acute bacterial gastroenteritis in The Netherlands [2–4]. In 2010, the incidence of laboratory-confirmed campylobacteriosis was 50 per 100,000 inhabitants, the highest ever recorded in the Dutch population since 1996. Up to 88% of these infections were acquired domestically. Hospitalization was required in approximately a quarter of laboratory-confirmed cases [5]. Most infections occur sporadically, with outbreak-related cases representing less than one percent of the total number of *Campylobacter* infections [6].

Apart from acute gastroenteritis, campylobacteriosis may lead to more severe, occasionally long-term, sequelae, such as Guillain-Barré syndrome, reactive arthritis, and irritable bowel syndrome [7,8], causing considerable morbidity and economic impact on the Dutch population [2,4,8]. *Campylobacter* spp. are commensally

widespread in the intestines of wild and domesticated animals, resulting in contamination of the environment, including water sources [9]. Although *Campylobacter* spp. are mostly perceived as food-borne pathogens, there is evidence for other transmission pathways, including direct and indirect contact with infectious animals, people, and environments [10–13].

Evidence of host-adapted *Campylobacter* strains exists [14]. However, the relative importance of each reservoir in zoonotic transmission remains unclear. Novel host-associated adaptive mutation and recombination events are frequent in *Campylobacter* spp., resulting in populations that are not strongly structured into differentiated clusters; thus, predicting host from genotype is challenging [14].

Several case-control studies have evidenced that consumption of chicken is an important risk factor for human campylobacteriosis [10–13,15]. Poultry and avian species in general are the preferential host for *Campylobacter* spp., and during processing retail poultry carcasses may become contaminated [6,9,16,17]. As *Campylobacter* strains of chicken origin may reach humans through pathways other than food [18], the consumption and handling of chicken may account for up to 40% of human infections, while up to 80% may be attributed to the chicken reservoir as a whole [9].

Case-control studies are insufficient for attributing human infections to the different reservoirs because they can only trace back to the points of exposure (e.g. food items consumed), which may not point to the original (amplifying) reservoirs because of cross-contamination. Attributing human infections to specific reservoirs is crucial to prioritize, implement, and measure the impact of targeted interventions [19]. Human *Campylobacter* infections can be attributed to specific reservoirs by estimating the extent of subtype sharing between strains isolated from humans and reservoirs [19]. Multilocus sequence typing (MLST) [20,21] is a typing methodology that is widely used internationally for this purpose [14,21–25]. MLST allows for the identification of genetic lineages in *Campylobacter* populations by indexing the variation present in seven housekeeping genes. A unique sequence pattern is assigned to a sequence type (ST), while closely related STs sharing the same alleles at different loci are considered as belonging to the same clonal complex (CC), the members of which possess a common ancestor [20]. Several modeling approaches can then be applied to MLST data to attribute *Campylobacter* strains from human cases to different reservoirs, e.g. [23].

With a focus on The Netherlands, the aims of this study were: 1) to attribute human *Campylobacter* infections to four putative animal reservoirs (chicken, cattle, sheep, and pig) and to the environment; 2) to combine the available case-control data [10] with the results of the attribution analysis to explore risk factors at the point of exposure for human campylobacteriosis caused by strains highly associated with the different reservoirs.

2. MATERIALS AND METHODS

2.1. Human data

We used *Campylobacter* data from the so-called CaSa study, a large case-control study on risk factors for sporadic salmonellosis and campylobacteriosis conducted in The Netherlands between April 2002 and April 2003. A detailed description of the methodology and results of the CaSa study is available elsewhere [10,26].

A total of 2858 *C. jejuni* and 257 *C. coli* cases were identified by the Dutch Regional Public Health Laboratories (RPHL) and assigned to species using molecular methods [27,28] at the Central Veterinary Institute (CVI) in Lelystad, The Netherlands. Cases were interviewed by means of a questionnaire sent by the RPHL together with the laboratory test results to the prescribing physician who forwarded these to the corresponding patient. After exclusion of cases who: 1) did not return or complete successfully the questionnaire (1679 cases); and 2) had a recent or unknown history of foreign travel, and/or lived outside The Netherlands (338 cases), 1019 *C. jejuni* and 79 *C. coli* cases were enrolled in the study.

Based on historic surveillance data of the number of *Campylobacter* and *Salmonella* infections in the RPHL service areas, the expected numbers of cases by age (0–4, 5–17, 18–29, 30–44, 45–59, and ≥60 years), sex, degree of urbanization (urban: >2500 addresses/km^2; urbanized: 500–2500 addresses/km^2; rural: <500 addresses/km^2), and season (April–June 2002, July–September 2002, October–December 2002, January–April

2003) were obtained. Controls were randomly selected from population registries within the RPHL service areas by frequency matching (aiming at two controls per case) according to the expected number of cases by age, sex, degree of urbanization, and season. A total of 10250 controls were approached in anticipation of an expected response rate of 25%. Of these, 3409 (33%) controls returned the postal questionnaire. After exclusion of controls who: 1) had travelled abroad (244 controls);or 2) did not provide reliable information (46 controls), 3119 controls were enrolled in this study.

Cases and controls were asked to fill in the aforementioned questionnaire to collect information regarding food consumption, kitchen hygiene, food processing, contact with animals, occupational exposure, history of travel, recreational water activity, medication use, history of chronic diseases, and contact with people with gastroenteritis. Questions covered the 7 days prior to symptoms onset (cases) or completion of the questionnaire (controls). Parents were asked to complete the questionnaire on behalf of their children. Missing values were handled using multiple imputation [29].

Isolates from 980 cases (919 *C. jejuni* and 61 *C. coli*) identified by the RPHL were successfully typed with MLST as described elsewhere [20,21]. Of these, 737 cases (696 *C. jejuni* and 41 *C. coli*) were cases enrolled in the study, as the other 243 typed cases were not eligible for enrollment because they had travelled abroad or did not return/complete successfully the questionnaire. Purification and sequencing of PCR products were done by Macrogen Inc, Korea. The software Bionumerics 5.10 was used to analyze sequence data.

Differences in relative frequencies of the five most frequently reported STs (ST-53, ST-50, ST-21, ST-48, and ST-45) and CCs (CC-21, CC-45, CC-206, CC-257, and CC-48) were examined for the variables age, sex, degree of urbanization, and season, using Pearson's $\chi 2$ test (α-level: 0.05).

2.2. Animal and environmental data

As only few Dutch *Campylobacter* reference strains typed with MLST were available for the animal reservoirs (232 strains) and for the environment (106 strains) [30] (Table 1), other reference strains from the United Kingdom (UK) [21], Scotland [25], and Switzerland [31] were used to supplement the Dutch ones. These data sets were identified among other published data sets from New Zealand, Australia, Curaçao, United States of America, and Finland (references available upon request), accessible in PubMLST (http://pubmlst.org/). The data sets from the UK, Scotland, and Switzerland were identified based on the similarity of the *C. jejuni* and *C. coli* ST frequency distributions of human isolates in these countries with those of human isolates in The Netherlands. The Euclidean distance was used as similarity metric in principal component analysis (PCA) [32]. The PCA revealed that the human isolates from The Netherlands were indeed most similar to the human isolates from the UK, Scotland, and Switzerland [33].

Table 1. Campylobacter strains used to feed the asymmetric island model for source attribution.

Country	Human	Chicken	Cattle	Sheep	Pig	Environment	Reference
The Netherlands	980†	210	9	0	13	106 (water)	[30] and data‡
United Kingdom	0	0	46	72	5	50 (sand)	[21]
Scotland	0	0	90	88	15	133 (wild birds)	[25]
Switzerland	0	0	23	0	100	0	[31]
Total	980	210	168	160	133	289	

†Obtained from the CaSa study [10].
‡Provided by the Central Veterinary Institute (CVI) in Lelystad, The Netherlands.

For the purposes of this study, the identified reservoir data [21,25,31], and those available for The Netherlands (i.e. [30] and additional data supplied by the CVI) were pooled and arranged in five groups: 1) chicken; 2) cattle; 3) sheep; 4) pigs; and 5) the environment (Table 1). Environmental strains were those sourced from water, sand, and wild birds, and were treated as a "reservoir" as well in the attribution analysis. Although the environment cannot be considered as a single amplifying host for *Campylobacter* spp. but only as a "pseudo-reservoir" collecting strains from a variety of different hosts, the STs found in environmental samples have hardly ever been found in other reservoirs [23,34]. Therefore, the environment was considered as a proxy for other unidentified reservoirs, putatively of primarily wildlife origin [23,34].

2.3. Attribution analysis

The Asymmetric Island (AI) model [22] was used to attribute human *Campylobacter* infections to the four putative animal reservoirs and to the environment. The AI model is a coalescent-based model derived from a generalization of the Wright's island model. It incorporates a Bayesian approach for modeling the genetic evolution and zoonotic transmission of the *Campylobacter* strains using their allelic profiles, accounting for relatedness among STs. The model estimates the mutation and recombination rates within the reservoirs, as well as the migration rates between reservoirs and from each reservoir to the human population. These migration rates are used to estimate the relative contribution of each reservoir to human infections [22]. By modeling the evolutionary processes of mutation and recombination, the AI model accounts for the occurrence of novel alleles, or novel combinations of alleles, in strains from humans that are unobserved in reservoir populations [22].

For every case, the AI model estimates a relative assignment posterior probability (*Pr*) to originate from each reservoir. The proportion of human infections attributed to a given reservoir is calculated as the sum of its *Pr* over cases divided by the total number of cases.

For each reservoir, differences in *Pr* were tested among age groups, degrees of urbanization, and seasons using the Kruskal-Wallis test (KW); and between genders using the Mann-Whitney U test (MW) (α-level: 0.05).

2.4. Risk factor analysis

We repeated the analysis of risk factors for human campylobacteriosis as previously applied [10], using the 737 cases typed with MLST and the 3119 controls enrolled. *C. jejuni* and *C. coli* infections were analyzed together. For preliminary significance testing, we assessed the association of 131 putative risk factors with *Campylobacter* infection using unconditional logistic regression with the matching variables and the level of education (categorized as: low = primary, lower vocational or lower secondary education; intermediate = intermediate vocational, intermediate secondary or higher secondary education; high = higher vocational and university education) included as covariates, which is the method of choice for frequency-matched data [35]. Factors showing a p-value lower than 0.10 for the association with the outcome in the single-variable analysis were selected for inclusion in a multivariable logistic regression model. A backward stepwise selection procedure was applied and variables with a p-value lower than 0.05 were retained in the final model. The population attributable risk (PAR) and the population preventable risk (PPR) of each significant factor were calculated based on multivariable odds ratios (OR) and the prevalence of exposure in cases. Similarly, confidence intervals of PARs and PPRs were derived from the confidence intervals of the multivariable ORs [10].

To investigate risk factors for human campylobacteriosis caused by *Campylobacter* strains highly associated with the different reservoirs, we constructed several logistic regression models that included separate subsets of cases assigned to the different reservoirs on the basis of the ranking of their estimated *Pr*s. The assignment of cases to the different reservoirs was performed similarly to previous case-studies [36,37]. The distribution of *Pr* for each reservoir was assessed and a cut-off point was determined to provide a reasonable balance between the number of cases assigned to each reservoir and the confidence as to their correct assignment derived by the highest possible *Pr*.

For infections of probable chicken, ruminant (cattle plus sheep), and environmental origin, separate logistic regression models that included only those cases with at least 50% probability (cut-off: $Pr \geq 0.50$) of originating from each of these reservoirs were constructed. For infections of probable chicken and ruminant origin, further logistic regression models were constructed for a range of other consecutive cut-off points at regular intervals of 0.05, from $Pr \geq 0.50$ to $Pr \geq 0.95$ for chicken, and from $Pr \geq 0.50$ to $Pr \geq 0.80$ for ruminants (Figure 1 and Table 2). The low numbers of the remaining cases did not allow for the construction of further models based on successive cut-off points. For the environment, it was only possible to construct a logistic regression model using the cut-off point of $Pr \geq 0.50$ (Figure 1 and Table 2) because there were too few cases with a higher *Pr* to enable consistent estimation.

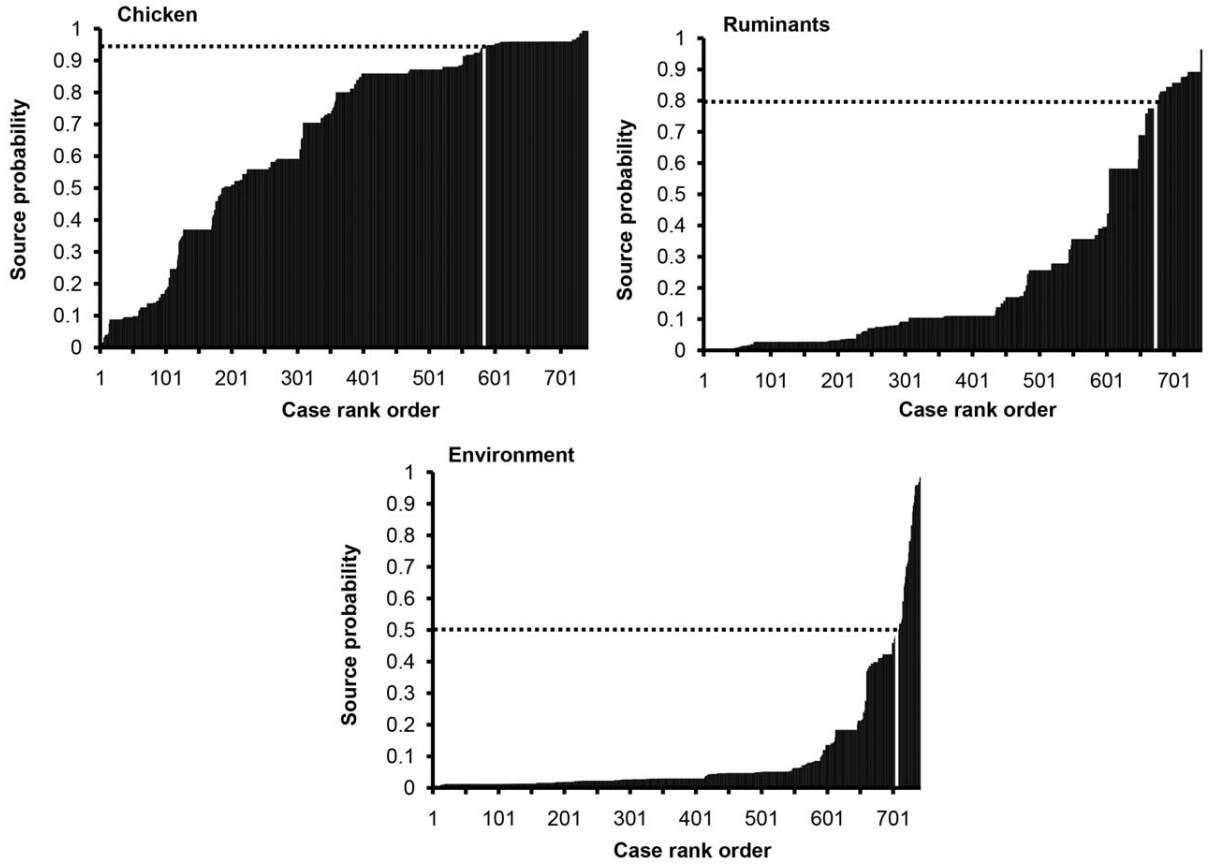

Figure 1. Rank ordered assignment source probability per human case (vertical columns). The white vertical columns indicate the cut-off points beyond which cases were selected for inclusion in the risk factor analysis. Cases are in ascending order according to the source probability to aid visualization.

The final cut-off points represented the best trade-off between the increasing *Pr* for a given reservoir (i.e. increase in reservoir specificity) and the decreasing number of cases includable in the models (i.e. decrease in statistical power and failure of the model to converge). For infections of probable chicken origin, 143 cases with a mean *Pr* for chicken of 0.96 (range: 0.95–0.99) were selected. For infections of probable ruminant origin, 67 cases with a mean *Pr* for ruminant of 0.87 (range: 0.80–0.96) were selected. Finally, for infections of probable environmental origin, 34 cases with a mean *Pr* for environment of 0.76 (range: 0.50–0.98) were selected (Figure 1 and Table 2).

For infections of probable pig and sheep origin, the construction of any regression model was technically possible, yet epidemiologically inappropriate, because there were no or just two cases with $Pr \geq 0.50$ for sheep and pig, respectively. Moving the cut-off point to a $Pr < 0.50$ for sheep and pig would have resulted in the inclusion of many cases nearly equally attributed to the different reservoirs, making the risk factor analysis unclear and relatively uninformative. For the risk factor analysis, cattle and sheep were thus combined into ruminants as done previously [36,37]. This option appears to be justified by the weak discrimination of *Campylobacter* strains from sheep and cattle when using MLST [14,22].

To explore if the risk factors of the multivariable logistic regression models differed according to age, sex, degree of urbanization, season, and level of education we also tested the significance of their interactions. The final multivariable logistic regression models were therefore expanded to include significant interaction terms.

For simplicity, only the results of the final multivariable regression models based on the aforementioned final cut-off points were presented. Although food and non-food related risk factors were estimated together, they were presented separately to improve readability of the tables. All regression models maximized to the *Pr* for a given reservoir showed an overall statistical significance (likelihood ratio χ^2 test, $p<0.05$) and an acceptable goodness-of-fit (Hosmer-Lemeshow test, $p>0.05$).

For all risk factor analyses, the controls were used as common comparison group. The matching variables and the level of education were always included as covariates in all regression models to control for confounding, as the *Pr*-based selection of cases

slightly skewed them from the controls with respect to these confounders. To support the accuracy of inferences of regression models with <5 cases per variable, bias-corrected bootstrap confidence intervals were also calculated (1000 replications) and compared with the standard ones [38]. As these confidence intervals did not differ significantly, the standard ones were reported. Statistical analyses were performed using STATA 11.2.

Table 2. Campylobacter sequence types of human cases assigned to chicken, ruminant, and the environment in the risk factor analysis.

Chicken[a]	Ruminants[b]	Environment[c]
44	19	350
227	22	447
230	38	508
290	61	586
353	104	587
354	206	637
400	270	696
443	403	710
584	432	861
606	475	1080
775	658	1539
801	1519	2123
859	2156	2130
875	2288	2151
883	2187	
978	3015	
1073	3130	
1191	4276	
1583	4279	
1600	4282	
1707	4300	
1728	4307	
1957	4308	
2034	4314	
2183		
2324		
2553		
2807		
2808		
2844		
2882		
2899		
3016		
4269		
4271		
4280		
4283		
4292		

a. 143 cases, mean Pr for chicken = 0.96; range: 0.95–0.99.
b. 67 cases, mean Pr for ruminants = 0.87; range: 0.80–0.96.
c. 34 cases, mean for environment Pr = 0.76; range: 0.50–0.98.

3. RESULTS

3.1. Human multilocus sequence types and clonal complexes

Overall, the 737 Campylobacter strains were assigned to 154 STs belonging to 28 CCs. Twenty-eight STs were unassigned to a previously identified CC. The frequency of genotypes was highly skewed, with ST-53, ST-50, ST-21, ST-48, and ST-45 accounting for more than a quarter of all isolates, and CC-21, CC-45, CC-206, CC-257, and CC-48 accounting for more than half of all isolates (Figure 2). The attribution analysis revealed that ST-50, ST-53, ST-48, and ST-45 were predominantly related to chicken, with a substantial contribution from cattle in ST-48 and ST-45 (Figure 3). ST-21 was mostly related to cattle and chicken (Figure 3). Chicken was also the predominant source for isolates belonging to CC-257, CC-206, CC-21, CC-45, and CC-48, but a substantial contribution from cattle was also found in CC-48 and CC-21, and from the environment in CC-45 (Figure 3).

For *C. jejuni*, the frequencies of STs and CCs followed the same ranking as the aforementioned ones; whereas for *C. coli*, the five most frequent STs were ST-825, ST-827, ST-1614, ST-854, and ST-1600, all belonging to CC-828, which accounted for 68% of *C. coli* isolates and was predominantly related to chicken (*Pr* = 0.69) and cattle (*Pr* = 0.16).

Significant age relationships were found for ST-53 (χ^2 test, p<0.001) and its CC, CC-21 (χ^2 test, p = 0.002). The highest relative frequency of ST-53 was found in children and adolescents (0–4 and 5–17 years, which accounted together for 48% of ST-53 isolates), whereas that of CC-21 (24% of CC-21 isolates) was found in young adults (18-29 years). ST-21 was significantly over-represented in urbanized areas (53%; χ^2 test, p = 0.036). Significant seasonal effects were found for ST-48 (χ^2 test, p = 0.023) and its CC, CC-48 (χ^2 test, p = 0.028), which showed the lowest relative frequencies in the spring (3% and 4%, respectively), peaked in the summer (43% and 40%, respectively) and had intermediate frequencies (23–31%) during autumn-winter months.

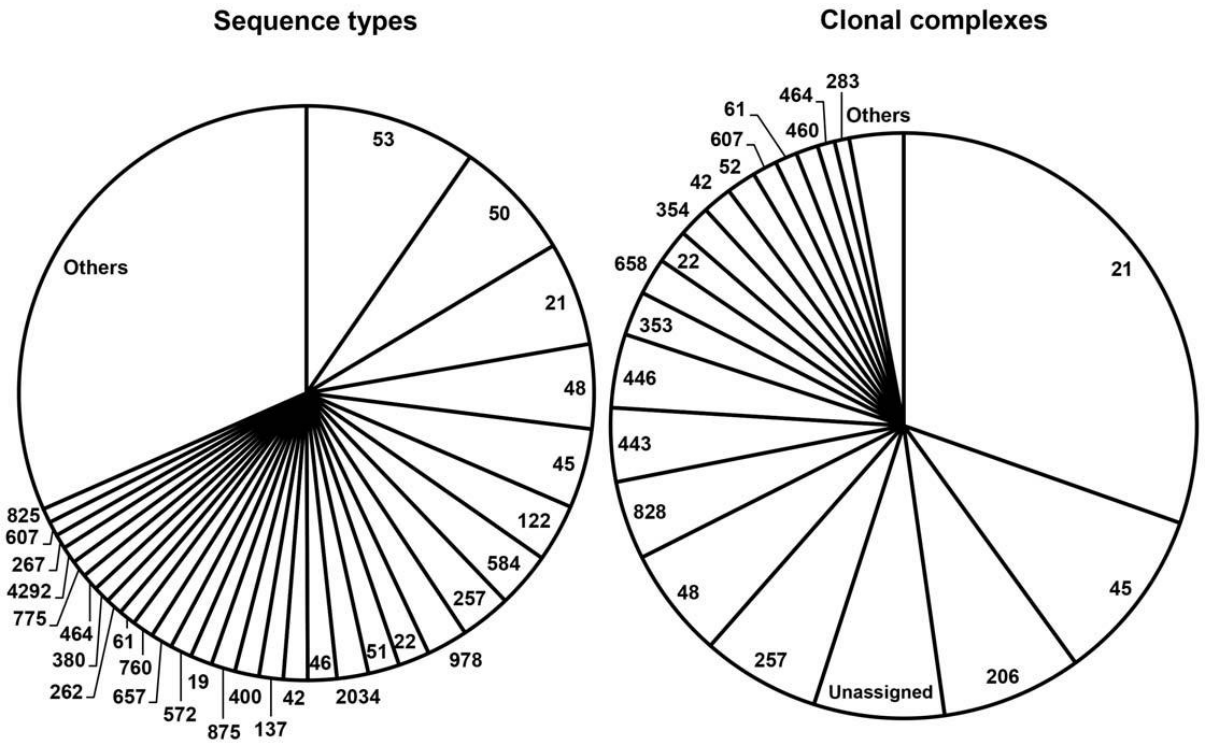

Figure 2. Human Campylobacter strains per clonal complex and sequence type assigned with MLST. The category 'others' includes clonal complexes and sequence types with less than five isolates.

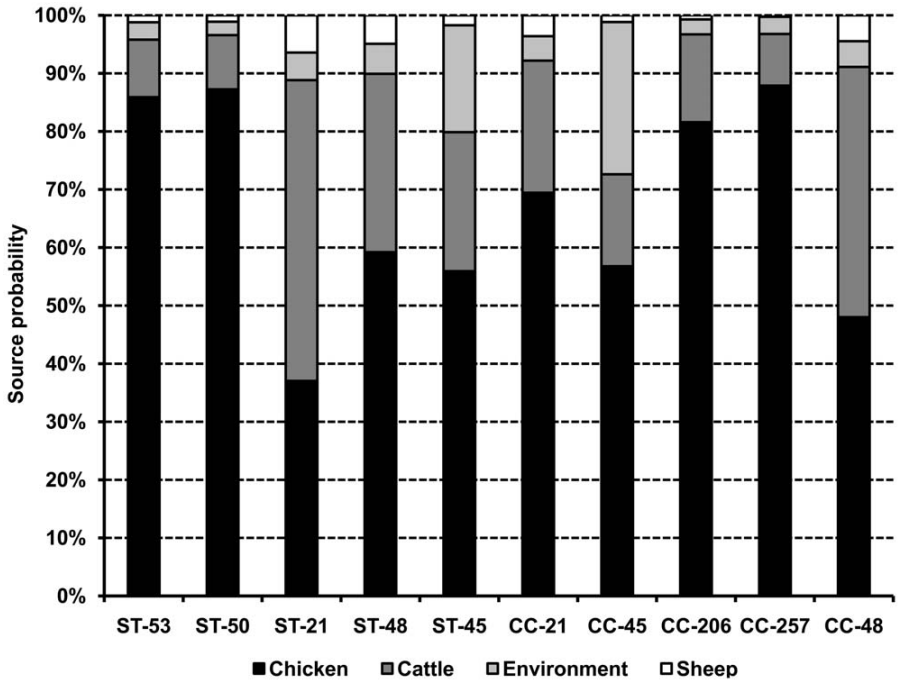

Figure 3. Attributed probability (%) for the five most represented sequence types and clonal complexes to originate from chicken, cattle, sheep, and the environment. The probability for pigs is not viewable because it is <1%.

3.2. Attribution of human infections

Overall, the AI model estimated that the majority of human infections (489; 66.2%) originated from chicken, followed by cattle (153; 20.7%), environment (74; 10.1%), sheep (19; 2.5%), and pigs (2; 0.3%). The 696 *C. jejuni* cases were attributed as follows: chicken, 66.1% (460 cases); cattle, 21.2% (148); environment, 10.2% (71); sheep, 2.4% (17); and pigs, 0.01% (<1) (Figure 4). The 41 *C. coli* cases were attributed as follows: chicken, 69.6% (29 cases); cattle, 12.2% (5); environment, 8.9% (3); sheep, 5.0% (2); pigs, 4.9% (2) (Figure 4).

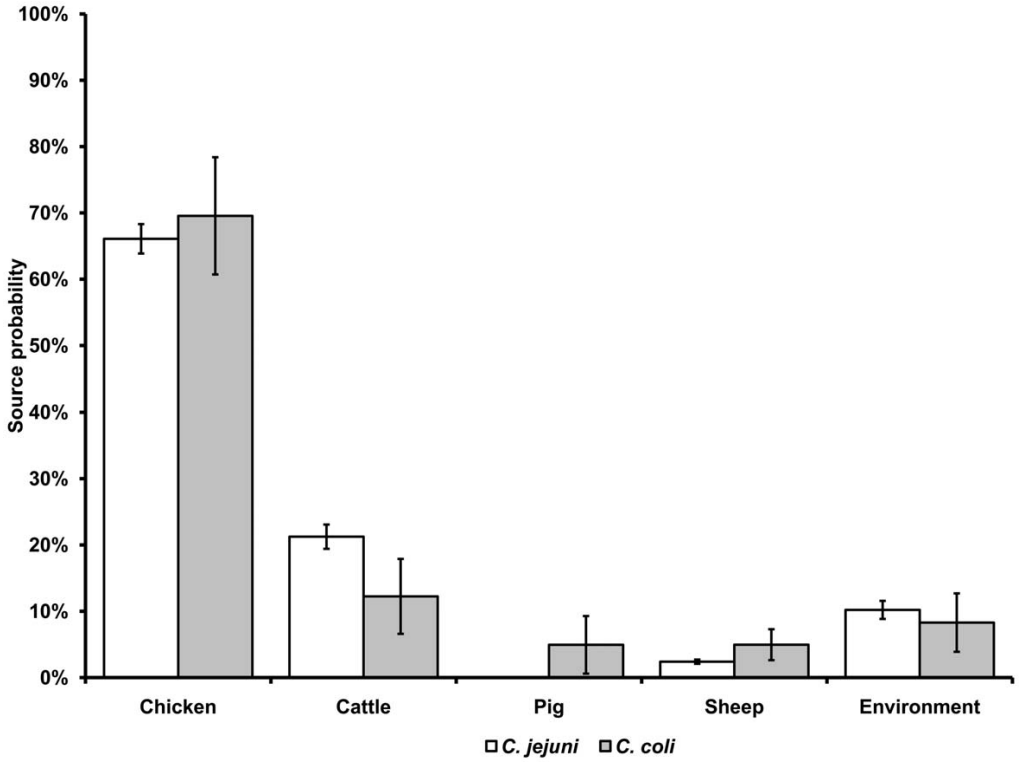

Figure 4. Overall mean probability (%) and 95% confidence interval for human *C. jejuni* (n = 696) and *C. coli* (n = 41) infections to originate from chicken, cattle, pig, sheep, and the environment.

The *Pr*s for cattle and pig were significantly different between *C. jejuni* and *C. coli* (cattle: MN, p = 0.001; pig: MN, p<0.001). Significant effects of urbanization were found for the chicken reservoir (KW, p = 0.023), which showed the highest median *Pr* (0.84) in cases from urbanized areas. A significantly (MN, p = 0.018) higher *Pr* for chicken was also found for young children aged 0–4 years living in urban areas (median *Pr*: 0.86; *n* = 15) compared with those living in rural areas (median *Pr*: 0.59; *n* = 33). A significant seasonal effect (KW, p = 0.020) was found in *Pr* for the environmental reservoir, which peaked in the spring followed by a trough in the summer and autumn, with a small peak in the winter.

3.3. General risk factors for campylobacteriosis

In contrast to the previous study [10], we used a smaller sample of cases and *C. jejuni* and *C. coli* were analyzed together. Nevertheless, the direction and strength of the factors associated with campylobacteriosis in the final multivariable model (Table 3 and Table 4) were comparable with the previous results [10]. With a PAR of 28%, consumption of chicken was the most important risk factor, followed by consumption of barbecued (18%) and undercooked (16%) meats, and eating in a restaurant (11%) (Table 3). However, a significantly higher risk was observed only when barbecued meat was consumed by patients living in non-urban areas (Table 3).Of the non-food risk factors (Table 4), strong associations were found for recent use of proton-pump inhibitors (22%), and having a chronic gastrointestinal disease (20%).

With a PPR of 34%, consumption of pasteurized dairy products other than milk and cheese (i.e. mostly yoghurt) was the most important protective factor, followed by consumption of chocolate (22%), pasteurized milk (15%), seafood (14%), fruit (13%), nuts (13%), meat in paste (8%), and salad (7%) (Table 3). Of the non-food protective factors, contact with dogs owned by other people was the most important one (8%) (Table 4).

Table 3. Multivariable odds ratios and percent PAR or PPR (and 95% confidence intervals) for food-related risk factors for human campylobacteriosis according to the attributed origin of the *Campylobacter* strain (chicken, ruminant, and the environment).

Risk factor (% imputed missing values*)	Overall[a]	Chicken[b]	Ruminants[c]	Environment[d]
Food consumption				
Chicken (1)	**1.5 (1.2–1.9) 28% (13–41%)**	**1.9 (1.2–2.9) 42% (14–60%)**	ns	ns
Beef (1)	ns	0.6 (0.4–0.9) 30% (7–44%)	ns	ns
Pork (2)	ns	0.7 (0.5–0.9) 16% (5–26%)	ns	ns
Tripe (1)	ns	ns	**4.0 (1.1–14.2) 12% (1–37%)**	ns
Game (0)	ns	ns	ns	**3.3 (1.4–7.8) 37% (10–64%)**
Undercooked meat (5)	**2.1 (1.6–2.7) 16% (10–23%)**	ns	ns	ns
Barbecued, grilled, or microwaved meat (5)	**18% (10–25%)**	ns	**63% (41–78%)**	ns
in urban areas	**1.2 (0.7–2.2)[ns]**	ns	0.8 (0.1–7.3)[ns]	ns
in urbanized areas	**1.7 (1.3–2.2)**	ns	**7.1 (3.2–15.6)**	ns
in rural areas	**3.0 (1.8–4.9)**	ns	**4.1 (1.3–14.2)**	ns
Meat in paste (croquette, meat roll, pastry) (5)	0.8 (0.6–1.0) 8% (0–13%)	ns	0.5 (0.2–0.8) 14% (1–22%)	ns
Pasteurized milk (1)	0.8 (0.6–0.9) 15% (6–29%)	ns	ns	ns
Pasteurized dairy other than milk or cheese (2)	0.6 (0.5–0.7) 34% (25–42%)	0.4 (0.3–0.7) 41% (20–48%)	0.5 (0.3–0.9) 37% (7–51%)	0.3 (0.1–0.8) 48% (13–61%)
Stir–fried vegetables (3)	ns	ns	ns	0.3 (0.1–0.9) 11% (2–16%)
Salad (2)	0.7 (0.6–0.9) 7% (2–9%)	ns	0.4 (0.2–0.9) 10% (2–13%)	ns
Fruit with peel (2)	0.7 (0.6–0.8) 13% (9–17%)	ns	ns	ns
Chocolate (2)	0.6 (0.5–0.7) 22% (17–28%)	0.5 (0.4–0.7) 22% (17–34%)	ns	ns
Nuts (3)	0.6 (0.5–0.8) 13% (6–16%)	ns	0.5 (0.3–0.9) 16% (0–22%)	ns
Seafood (4)	0.5 (0.4–0.7) 14% (8–16%)	0.6 (0.4–0.9) 13% (3–20%)	ns	ns
Eating habits				
Eating in a restaurant (0)	**1.3 (1.1–1.6) 11% (4–20%)**	ns	ns	ns
Vegetarian diet (0)	0.4 (0.2–0.9) 1% (0–1%)	ns	ns	ns
Eating chicken once a month or less (3)	ns	ns	**1.7 (1.0–2.9) 25% (1–47%)**	ns
Kitchen hygiene				
Not cleaning a knife when using it for raw meat and other foods (1)	**1.7 (1.1–2.6) 4% (6–9%)**	ns	ns	ns
Washing hands before food preparation (0)	0.6 (0.4–0.9) 1% (0–2%)	ns	ns	ns

Multivariable odds ratios are also adjusted for age, sex, degree of urbanization, season, and level of education. PAR (population attributable risk) and PPR (population
preventable risk) are based on the multivariable odds ratios. Risk factors are in bold, protective factors in normal font.
ns = not significant (p.0.05).
*Fraction of imputed missing values in the whole dataset.
a. 737 cases; mean Pr for chicken = 0.66 (range: 0.00–0.99); mean Pr for ruminants = 0.23 (range: 0.00–0.96); mean Pr for environment = 0.10 (range: 0.00–0.98).
b. 143 cases; mean Pr for chicken = 0.96; range: 0.95–0.99.
c. 67 cases; mean Pr for ruminants = 0.87; range: 0.80–0.96.
d. 34 cases; mean Pr for environment = 0.76; range: 0.50–0.98.

3.4. Risk factors for chicken-associated campylobacteriosis

For chicken-associated campylobacteriosis, consumption of chicken was the most important risk factor (PAR 42%) (Table 3), followed by recent use of proton-pump inhibitors (34%), having a chronic gastrointestinal disease (12%), and contact with people with gastroenteritis symptoms outside the household (10%) (Table 4). Important protective factors were consumption of yoghurt (PPR 41%), beef (30%), pork (16%), and seafood (13%) (Table 3).

3.5. Risk factors for ruminant-associated campylobacteriosis

With a PAR of 63%, consumption of barbecued meat was the most important risk factor for ruminant-associated campylobacteriosis (Table 3). However, the

risk posed by the consumption of barbecued meat was significantly higher only for patients living in non-urban areas. Other important risk factors were: consumption of tripe (12%), eating chicken rarely, i.e. once a month or less (25%) (Table 3), recent use of proton-pump inhibitors (34%), and occupational exposure to animals (17%) (Table 4). Important protective factors were consumption of yoghurt (PPR 37%), nuts (16%), and meat in paste (14%) (Table 3).

Table 4. Multivariable odds ratios and percent PAR or PPR (and 95% confidence intervals) for non-food related risk factors for human campylobacteriosis according to the attributed origin of the Campylobacter strain (chicken, ruminant, and the environment).

Risk factor (% imputed missing values*)	Overall[a]	Chicken[b]	Ruminants[c]	Environment[d]
Contact with animals				
Contact with dog(s) owned by other people (3)	0.6 (0.5–0.8) 8% (4–10%)	ns	ns	ns
Contact with pets and/or farm animals outside the household (1)	ns	ns	ns	0.4 (0.2–1.0) 17% (1–22%)
Ownership of several dogs, at least one dog < 1 year–old (0)	**2.5 (1.1–5.8)** **2% (1–7%)**	ns	ns	ns
Ownership of several dogs, all dogs > 1 year–old (0)	ns	ns	ns	**3.5 (1.0–12.0)** **33% (1–54%)**
Ownership of cat(s) (1)	**1.4 (1.2–1.8)** **10% (5–17%)**	ns	ns	ns
Recent use of medication				
Antibiotics (0)	0.4 (0.2–0.8) 1% (0–2%)	ns	ns	ns
Proton–pump inhibitors (0)	**3.7 (2.5–5.5)** **22% (14–33%)**	**4.7 (2.4–9.1)** **34% (11–53%)**	**5.7 (2.2–16.3)** **34% (11–58%)**	ns
Other				
Swimming in a domestic swimming pool (0)	ns	ns	ns	**28% (2–64%)**
in the spring season	ns	ns	ns	**16.8 (2.6–107.6)**
in the summer, winter or autumn seasons	ns	ns	ns	**2.5 (0.4–14.4)**[ns]
Contact with people with gastroenteritis symptoms outside the household (3)	**1.5 (1.1–2.1)** **6% (1–12%)**	**1.8 (1.1–3.0)** **10% (1–23%)**	ns	**3.4 (1.3–8.7)** **35% (6–63%)**
Having a chronic gastrointestinal disease (0)§	**2.4 (1.8–3.2)** **20% (13–28%)**	**1.8 (1.1–3.1)** **12% (2–27%)**	ns	**5.0 (2.1–12.1)** **50% (22–74%)**
Occupational exposure to animals (0)	ns	ns	**3.2 (1.2–9.0)** **17% (2–41%)**	ns

Multivariable odds ratios are also adjusted for age, sex, degree of urbanization, season, and level of education. PAR (population attributable risk) and PPR (population
preventable risk) are based on the multivariable odds ratios. Risk factors are in bold, protective factors in normal font.
ns = not significant (p.0.05).
*Fraction of imputed missing values in the whole dataset.
a. 737 cases; mean Pr for chicken = 0.66 (range: 0.00–0.99); mean Pr for ruminants = 0.23 (range: 0.00–0.96); mean Pr for environment = 0.10 (range: 0.00–0.98).
b. 143 cases; mean Pr for chicken = 0.96; range: 0.95–0.99.
c. 67 cases; mean Pr for ruminants = 0.87; range: 0.80–0.96.
d. 34 cases; mean Pr for environment = 0.76; range: 0.50–0.98.
§Includes Crohn's disease, irritable bowel disease (IBD), irritable bowel syndrome (IBS), or celiac disease.

3.6. Risk factors for environment-associated campylobacteriosis

Consumption of game was the only food-related risk factor for campylobacteriosis of probable environmental origin (PAR 37%) (Table 3). Other important risk factors were: having a chronic gastrointestinal disease (50%), contact with people with gastroenteritis symptoms outside the household (35%), swimming in a domestic swimming pool (28%), and ownership of several adult dogs (33%) (Table 4). However, a significantly higher risk was observed only when patients swam in a domestic swimming pool during the spring (April–June), but not during the other seasons (the risk of swimming during the summer, autumn, and winter months was equally insignificant; thus, these strata were combined, Table 4). Important protective factors were consumption of stir-fried vegetables (PPR 11%) (Table 3) and contact with pets and/or farm animals outside the household (17%) (Table 4).

4. DISCUSSION

This is the first case-control study in which risk factors at the point of exposure for

human campylobacteriosis are investigated in relation to the attributed reservoirs based on MLST profiles. Previous studies [36,37] examined risk factors for reservoir-associated campylobacteriosis in a similar, albeit more limited, way, as only a small number of risk factors about demographic characteristics (e.g. age, sex, resident location, etc.) were investigated and a case-case approach was used.

4.1. *Campylobacter* multilocus genotypes

Campylobacter populations are regarded as genetically highly diverse, even when considering their core-genome using MLST [9]. With 154 STs identified among 737 human cases, our results indicate that considerable variety exists also in the Dutch Campylobacter population. Rare STs were also considerably represented, as STs occurring once accounted for 46% of all STs. Besides this large variety, there was some evidence indicating that certain genotypes can emerge and predominate in specific age groups, areas, and seasons, although most of the commonest genotypes were broadly distributed and recurrent over time.

The main STs and CCs identified here have been reported worldwide [21,23,25,39–44] and were typical of previous reports from The Netherlands [20,30]. Most of these studies, however, were geographically and temporally limited; thus, the extent to which the predominant genotypes, both in humans and reservoirs, correspond to stable geographical structuring or to a transient expansion could not be investigated.

In our study, CC-21 was the most represented CC and was predominated (32%) by ST-53, which was also the most common ST in the whole data set. Although both CC-21 and ST-53 were primarily attributed to chicken, they were over-represented among cattle isolates in Scotland [25] and Finland [40], and among cattle and chicken isolates in the UK [21] and multi-country collections [43]. To a lesser extent, there is also evidence for sheep and environment to be involved [21,22,25,40,43,44]. This also applies to the ubiquitously sourced ST-50, ST-21, ST-48, and ST-45, although they seem to be predominant in chicken (ST-50 and ST-45) and cattle (ST-21 and ST-48) [21,23,40,43]. Differences in host preference among *Campylobacter* genotypes observed in this study may be due to niche adaptation, geographic separation, host-related factors (e.g. immunity, behaviors with respect to potential exposures, etc.), or barriers to genetic exchange [40].

4.2. Attributed reservoirs of human campylobacteriosis

Chicken was estimated to be the most important reservoir of human campylobacteriosis in The Netherlands, accounting for approximately 66% of infections. This is in line with other studies conducted in industrialized countries using the AI model [22–24]. The proportion of cases attributable to chicken, however, varied considerably among these studies (56% [22] and 76% [23,24]). Besides variations in local epidemiology, such divergences are mostly due to the consideration of different reservoirs, which may affect the proportions of attributed infections. For instance, Mullner *et al*. [23] did not consider pigs; Sheppard *et al*. [24] kept wild birds, environment, and turkey as separated sources; Wilson *et al*. [22] included also rabbit and kept wild birds, sand, and water separated.

We found that *Pr*s for pig and cattle were significantly different between *C. jejuni* and *C. coli* strains. A higher *Pr* for cattle was found in *C. jejuni* compared with *C. coli*, whereas a higher *Pr* for pig was found in *C. coli* compared with *C. jejuni*. This supports evidence indicating that *C. jejuni* is more prevalent than *C. coli* in cattle and that the inverse situation holds for pigs [9]. We also found that chicken was the major reservoir for campylobacteriosis in young children living in urban areas compared with their rural counterparts, for which cattle seemed to be more important, although the difference for cattle was not clearly significant (data not shown). The same finding was previously observed in Scotland [25] and New Zealand's North Island [37], supporting the hypothesis that the main source of campylobacteriosis for young children depends on residence location: chicken (consumption) is a more important source of infection in urban dwellers, while infection from cattle seems to be more likely to occur in rural areas, possibly via environmental pathways [25]. In The Netherlands, cattle density has also been associated with an increased risk for Shiga toxin-producing *Escherichia coli* (STEC) O157 infection in young children living in rural areas [45]. Together these results suggest

that the risk of encountering and becoming diseased with enteropathogens putatively shed by cattle is considerable in young children living in rural areas.

A significant seasonal effect was found for the environmental reservoir. *Campylobacter* is widespread in the environment where it generally gives clues to recent fecal contamination, agricultural run-off, and sewage effluent [46]. Although intestinal carriage of *Campylobacter* is ubiquitous in animals, the environmental contamination varies seasonally depending on factors such as stress, changes in diet, and indoor/outdoor housing of animals [46]. The significant seasonal pattern of campylobacteriosis of probable environmental origin may reflect both the year-round variation in *Campylobacter* die-off rates in varying environments and the increased propensity of people for outdoor recreational activities, especially water activities, during the warm season, which may entail transmission from outdoor-reared animals and so far unidentified wildlife reservoirs.

4.3. Reservoir-specific risk factors for campylobacteriosis

While the attribution analysis quantified the relative contributions of the considered reservoirs to human infections, the risk factor analysis identified the excess risks for infections that were highly associated with these reservoirs, allowing for the identification of the possible pathways by which *Campylobacter* infection may be acquired from a given reservoir, as well as their quantification in terms of PAR. For instance, only up to 42% (14–60%) of the highly chicken-associated infections could be ascribed to consumption of chicken, supporting the hypothesis that a considerable part of infections originating from chicken is acquired by pathways other than food, such as the environment [18], or by cross-contamination to commodities, utensils, and foods other than chicken [47]. Indeed, it has been suggested that sporadic campylobacteriosis is more likely to occur because of cross-contamination from raw poultry products than because of consumption per se [12].

Some factors may be significantly associated with infections attributed to a given reservoir just because these infections have a residual contribution from reservoirs other than those to which they were attributed. Although the selection of cases for the risk factor analysis was based on the highest possible *Pr*s, residual attributions were 4%, 13%, and 24% in chicken-, ruminant-, and environment-associated infections, respectively. Nevertheless, all risk factors were associated in an epidemiologically plausible way according to the reservoir in question. For instance, consumption of chicken was a risk factor for infections of chicken origin whereas the consumption of beef and pork appeared to protect against chicken-associated infections. Plausibly, a person may be “protected” against infection with the most chicken-associated *Campylobacter* strains when exposed to reservoirs other than chicken, such as pig and cattle. Furthermore, consumption of tripe, barbecued meat, and seldom or never consumption of chicken were risk factors for infections attributed to ruminants. Possibly, people consuming chicken rarely may consume meats and other edible products from ruminants more frequently. Although we did not have any information about the type of meat cooked at the barbecue, it is clear that red meats are more likely to be consumed rare when barbecued, and thus more likely to harbor viable *Campylobacter* due to incomplete cooking. Besides undercooking, barbecuing usually provides many opportunities for re- and cross-contamination. The fact that the risk posed by barbecued meat was higher in patients living in non-urban areas, and insignificant in those living in urban ones, is supportive of the aforementioned hypothesis that ruminant-associated infections are more likely to occur in the countryside [25,37]. Working with animals was also a risk factor for infections attributed to ruminants, supporting another hypothesis stating that these infections may be acquired, to a considerable extent, through animal contact rather than food [23,24].

Consumption of game and swimming in a domestic swimming pool increased the risk for infections of probable environmental origin. In our study, the environmental reservoir included strains from wild birds, water, and sand. Although water and sand cannot be considered as amplifying hosts, they can act as vehicles delivering an exposure possibly from primary wildlife reservoirs [23,34]. In The Netherlands, *Campylobacter* is commonly found in recreational water [48] and domestic swimming pools mainly consist of temporary outdoor inflatable swimming pools of limited capacity, which can easily become

contaminated by bird feces. Moreover, it is likely for cleaning and maintenance procedures of swimming pools (e.g. water chlorination) to be less strictly applied in a domestic context. However, we found that the risk posed by domestic swimming pools was only significant in the spring but not in the other seasons. This is in accordance with our other finding indicating that the importance of the environmental reservoir varies seasonally, with a major peak in the spring. While most (outdoor) swimming pools are unusable during autumn-winter months, several British studies (reviewed by Jones [46]) have evidenced that there is a negative correlation between hours of sunshine and *Campylobacter* presence in recreational water, with significantly lower isolation rates in the summer compared to the other seasons corresponding to elevated ultraviolet radiation levels and higher temperatures, two conditions that greatly affect the survival of *Campylobacter* spp. outside the host. Moreover, it is possible that swimming pools are cleaned more frequently in the summer as a result of their more frequent use, or that other exposures and reservoirs play competitively a more prominent role in the summer.

We found that recent use of proton-pump inhibitors, having a chronic gastrointestinal disease, and contact with people with gastroenteritis symptoms outside the household were risk factors for infections attributed to different reservoirs. It is conceivable that the neutralizing effect of proton-pump inhibitors on gastric acidity may enhance *Campylobacter* survival during its passage through the stomach and that a disturbed intestinal function may facilitate infection [10]. However, it is also possible that *Campylobacter* infections are more likely to be diagnosed in people affected by chronic gastrointestinal diseases, as these people may be under more frequent medical attention (closer surveillance) and diagnostic thoroughness. Person-to-person transmission is uncommon for campylobacteriosis but it probably occurs with no particular preference for the primary reservoir of the *Campylobacter* strain involved. However, it is worth mentioning that this risk was particularly pronounced for *Campylobacter* strains of probable environmental origin. Considering that our environmental strains were sourced from water and sand among others, and that person-to-person transmission seems particularly important in children [10], it can be speculated that sand (particularly the one in playground sand-boxes) and recreational water can act as a vehicle for transmission among humans as well.

Consumption of several non-meat foods, including fruits, vegetables, dairy (mostly yoghurt), and seafood, were protective against infections attributed to different reservoirs. It is believed that these foods may have genuinely beneficial effects on general health by inhibiting bacterial growth, enhancing general immunity to infection, and altering the intestinal flora in a way that prevents infection [10,12,13,15].

4.4. Limitations and possible sources of bias

We supplemented Dutch data of reservoirs with data from other countries, an approach that has previously been applied [14,24], but could introduce bias in the attribution estimates. However, it has been shown that the association of multilocus genotypes with specific hosts transcends geographical variations [49]. Therefore, although greater accuracy of attribution estimates is possible with reference data closely sampled in space and time, these are not essential, and reference data from other regions can be used where local data are not available. To address this, we performed a PCA on human data from different countries to identify the corresponding reservoir data that were expected to be close to those present in The Netherlands in 2002–2003 [32]. The underlying assumption was that, if the ST frequency distribution of the human population of The Netherlands resembles that of the human population from another study, then the Dutch reservoir data may well resemble the reservoir data from that study. Apart from the reservoirs and their ST frequency distributions, consumption patterns and exposure pathways were assumed to be comparable, an assumption that has some plausibility among northern European countries. A detailed description of the results of the PCA will be provided in another manuscript that is in preparation.

This study was restricted to *C. jejuni* and *C. coli*. These two species, however, account for up to 98% of infections characterized at species level in The Netherlands [10]; thus, the impact of the other species on attribution estimates was expected to be minimal. It is clear that when exposures are aggregated for *C. jejuni* and *C. coli*

infections, the contribution of risk factors primarily associated with *C. coli* may be masked by the numerical superiority of *C. jejuni*. However, cases were split according to *Pr*, and *C. jejuni* and *C. coli* could potentially originate from a same reservoir. The primary outcome of interest was thus to explore reservoir-specific risk factors for campylobacteriosis rather than accounting for *Campylobacter* species-specific risk exposure characteristics. Another limitation concerns the residual contribution to *Pr* by reservoirs other than those to which infections were attributed. To address this in the risk factor analysis, we constructed regression models (when not limited by sample size) that were restricted to subsets of cases with the highest possible *Pr* for each reservoir. The residual contribution, although minimized, creates "noise" which could have masked or diluted some associations, or led to some additional associations, in the risk factor analysis. The latter option could be the case of the ownership of several adult dogs as a risk factor for environment-associated strains. Nonetheless, dogs are often tested positive for *Campylobacter* spp. and it has been suggested that dogs housed in group have a higher prevalence, possibly due to dog-to-dog transmission [50]. Moreover, dog owners may be particularly exposed to *Campylobacter* strains of environmental origin while walking their dogs, and adult dogs living in a group are also more likely to have (unsupervised) outdoor access and can therefore act as a vehicle for *Campylobacter* strains of environmental origin, possibly acquired upon ingestion of contaminated water, predation, necrophagy, and coprophagy. While dog ownership increases the risk for environment-associated infections, contacting an animal outside the household appears to be protective. We speculated that contacting animals other than their own encourages individuals to undertake protective actions, such as hand washing.

Many isolates from the cases included in the previous case-control study [10] were no longer viable and could not be cultured and typed with MLST for the purposes of this study. This could be due to underlying differences in survival among the different *Campylobacter* strains. However, we were able to replicate the results of the previous study [10], suggesting that our subset of cases was not biased and that the non-typed isolates were missed at random.

It has been postulated that repeated exposure to different *Campylobacter* strains may lead to sufficient immunity to provide protection against (severe) clinical illness [1,51]. In case-control studies, this protective immunity would lead to misclassification, as some controls could have been infected with *Campylobacter* spp. asymptomatically. As cases were identified by passive surveillance, they were likely to represent the most severe, symptomatic infections that occurred in the population. Thus, the identified risk factors especially represent risk factors for severe campylobacteriosis. Other concerns in case-control studies are recall and selection bias. Specifically in this study the recall period for cases was longer than for controls, and controls returning the postal questionnaire could be particularly motivated people with a generally healthier lifestyle, a fact that provides an alternative explanation of why, for example, eating fruits and vegetables were protective factors. Nevertheless, similarly to the previous study [10], these possible biases were explored by conducting multiple imputation checks and case-case analyses (data not shown), which revealed that both recall and selection bias had limited impact on our results.

5. CONCLUSIONS

A number of case-control studies have explored risk factors for *Campylobacter* infection while other studies have used MLST data to attribute *Campylobacter* infections to animal or environmental reservoirs, as well as used a case-case approach to characterize the risk of becoming infected with *Campylobacter* strains of different origins. Our study attempts to bridge this gap by exploring risk factors at the point of exposure for campylobacteriosis of different origins, using a combined case-control and source attribution analysis.

Our results lend weight to the suggestion that human campylobacteriosis in The Netherlands could greatly be reduced by focusing interventions on chicken and cattle. Chicken seems to be the major reservoir of campylobacteriosis for people living in cities, whereas cattle seems to be more important in their rural counterparts. The importance of the chicken and cattle reservoirs, however, was only partially consistent with food-borne transmission, as alternative pathways, such as direct contact and environmental contamination, do play a role as well,

particularly for infections attributed to ruminants.

This study showed that risk factors for *Campylobacter* infection depend upon the attributed reservoir and that the exposure may plausibly direct to the original reservoir when considering those *Campylobacter* strains that are indeed highly associated with the reservoir in question. Combining epidemiological and genotype-based source attribution data was helpful in enhancing risk factor identification and characterization for human campylobacteriosis and in providing a valuable approach for supporting and generating hypotheses. In a broader perspective, our results also indicate that the general concept of genotype-based source attribution modeling for campylobacteriosis makes sense epidemiologically. More Dutch reference strains from other animal reservoirs, such as dogs and cats, as well as different categorizations of food-producing animals, will provide a better discrimination of *Campylobacter* reservoirs and possibly stimulate novel epidemiological insights towards reservoir-specific risk factors and transmission pathways for human campylobacteriosis.

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Chapter 7

Increased risk for *Campylobacter jejuni* and *C. coli* infection of pet origin in dog owners and evidence for genetic association between strains causing infection in humans and their pets

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Increased risk for *Campylobacter jejuni* and *C. coli* infection of pet origin in dog owners and evidence for genetic association between strains causing infection in humans and their pets

ABSTRACT

We compared *Campylobacter jejuni/coli* multilocus sequence types (STs) from pets (dogs/cats) and their owners and investigated risk factors for pet-associated human campylobacteriosis using a combined source attribution and case-control analysis. 132/687 pet stools were *Campylobacter*-positive, resulting in 499 strains isolated (320 *C. upsaliensis/helveticus*, 100 *C. jejuni*, 33 *C. hyointestinalis/fetus*, 10 *C. lari*, 4 *C. coli*, 32 unidentified). 737 human and 104 pet *C. jejuni/coli* strains were assigned to 154 and 49 STs, respectively. Dog, particularly puppy, owners were at increased risk of infection with pet-associated STs. In 2/68 cases versus 0.134/68 expected by chance, a pet and its owner were infected with an identical ST (ST-45, ST-658). Although common sources of infection and directionality of transmission between pets and humans were unknown, dog ownership increased significantly the risk for pet-associated human *C. jejuni/coli* infection and isolation of identical strains in humans and their pets occurred significantly more often than expected.

1. INTRODUCTION

Dogs and cats are popularly kept for companionship in industrialized countries. In the Netherlands (16.5 million population), there were approximately 1.5 million dogs and 3 million cats in 2010, with 21% and 34% of households owning at least one dog or cat, respectively [1].

Ownership of dogs and cats (hereafter referred to as pets) is beneficial to the owner's psychological and physical health, promoting a less sedentary lifestyle, emotional protection and social interaction [2]. However, pets frequently enjoy great freedom in their owner's home, which may include occupying their owner's bed [3]. A recent survey in the Netherlands indicated that among 159 households with pets, 45% of the dogs and 62% of the cats were allowed on the bed [4]. Half of the owners allowed the dogs to lick their face and 45% allowed the cats to jump onto the kitchen sink. This close interaction between pets and humans raises concerns regarding the potential zoonotic risks.

Campylobacteriosis, a frequently occurring foodborne infection in the Netherlands [5], is potentially transmissible between pets and humans via the faecal-oral pathway [6]. Owning a pet, especially a puppy, has been identified as a risk factor for *Campylobacter jejuni* and *C. coli* infection [7]. *Campylobacter* may cause both symptomatic and asymptomatic infections in pets [6], with similar isolation rates in both diarrhoeic and healthy animals [8, 9]. Symptomatic infections mainly occur in young animals and are often caused by *C. jejuni* [6].

Although infection in pets and humans from a common source is possible, there is also evidence for transmission between pets and humans from molecular studies of *C. jejuni* using amplified fragment length polymorphism [10], and pulsed-field gel electrophoresis [11].

Multilocus sequence typing (MLST) [12] is widely used for the purposes of source attribution of human *Campylobacter* infections [13–17], and has not yet been used to investigate the genetic relatedness of strains from pets and their owners. In this study, we investigated MLST profiles of *C. jejuni* and *C. coli* strains isolated from pets and their owners. We also estimated the probability that these human strains originated from pets or other putative reservoirs by conducting a source attribution analysis. Finally, we combined

case-control and source attribution data to explore risk factors at the point of exposure for human campylobacteriosis of probable pet origin.

2. METHODS

2.1. Epidemiological data

Data from the CaSa study, a large case-control study on risk factors for human campylobacteriosis and salmonellosis conducted in the Netherlands between April 2002 and April 2003, formed the basis of this study. A detailed description of the CaSa study is available elsewhere [7,18].

A total of 2858 *C. jejuni* and 257 *C. coli* laboratory-confirmed human cases were identified by the Dutch Regional Public Health Laboratories (RPHL) through passive surveillance of diarrhoeic patients seeking for medical attention. Isolates were sent to the Central Veterinary Institute (CVI) in Lelystad, the Netherlands, for *C. jejuni* and *C. coli* species determination using molecular methods [19,20]. Cases that did not return or complete successfully the questionnaire used to collect epidemiological information (1679 cases, 54%) and/or had a recent or unknown history of travel and/or lived abroad (338 cases, 11%), were excluded, leaving 1019 *C. jejuni* and 79 *C. coli* cases (35%) enrolled in the study. Isolates from 737 (67%) enrolled cases were typed using MLST [12].

Controls were randomly selected from population registries within the RPHL service areas by frequency matching (aiming at two per case) according to the expected number of *Campylobacter*/*Salmonella* cases (based on historic surveillance data) by age (0–4, 5–17, 18–29, 30–44, 45–59, ≥60 years), gender, urbanization degree (urban: >2500, urbanized: 500-2500, rural: <500 addresses/km^2), and season (April–June 2002, July–September 2002, October–December 2002, January–March 2003). A total of 10250 controls were approached and 3409 (33%) returned the questionnaire. After exclusion of controls that travelled abroad and/or did not complete successfully the questionnaire, 3119 (91%) controls were enrolled.

Cases and controls were asked to fill in a questionnaire regarding food consumption, kitchen hygiene, contact with animals, occupation, recreational activity, medication use, history of chronic diseases, and contact with people with gastroenteritis. Questions covered the seven days prior to symptoms onset (cases) or questionnaire completion (controls). Parents completed the questionnaire for their children. Missing values were handled using multiple imputation [21].

Cases owning a pet were asked to submit a faecal sample of their pets to be tested for *Campylobacter* spp. and typed with MLST if they were positive for *C. jejuni* or *C. coli* using the same methods as for human cases. Pet faecal samples were submitted by mail: an envelope was sent to the owners including a container without transport medium together with instructions for collecting and returning the material. A minimum amount of faeces was requested as to minimize the die-off due to dry conditions. Samples were transported overnight and processed the following day. A total of 687 pet faecal samples were submitted. The sample origin (dog or cat) could be determined for only 424 (62%) samples (315 from dogs and 109 from cats) because this information was not always indicated by the submitting owner, nor could it be inferred from the patient's questionnaire because of cohabitation of dogs and cats in the same household. A median of 32 days (interquartile range (IQR) = 13, min-max: 10–71) separated human and pet faecal sampling. The software Bionumerics 5.10 was used to analyse sequence data. The expected probability (P) of finding an identical sequence type (ST) *x* in a human case and a pet living in the same household by purely random chance was calculated as: P(human ST = pet ST) = $\sum_x${P(human ST = *x* | human sample = positive for *C. jejuni* or *C. coli*) × [P(pet ST = *x* | pet sample = positive for *C. jejuni* or *C. coli*) × *M*]}, where $\sum_x$ is the summation over all STs found in both pets and humans and *M* is the overall prevalence of *C. jejuni* and *C. coli* found in our pet sample.

The Proportional Similarity Index (PSI) [15] was used to measure the similarity between sequence type (ST) frequency distributions of pets, pet owners, and non-pet owners. PSI values range between one (identical frequency distributions) to zero (distributions with no common ST).

2.2. Attribution analysis

MLST data from *Campylobacter* strains obtained from chicken, cattle, sheep, pig, pets, and the environment (water, sand, and wild birds) were supplied by the CVI and

supplemented with other data from the UK [12], Scotland [17], and Switzerland [22] to provide a representative dataset for each reservoir (Table 1). As environmental strains have rarely been found in the other reservoirs [15], they were treated as proxy for other unidentified reservoirs, putatively of wildlife origin [15].

The Asymmetric Island (AI) model, a Bayesian population genetics algorithm for modelling *Campylobacter* evolution and transmission [14], was used to estimate the posterior probability (*Pr*) for each human ST to originate from the considered reservoirs. Two separate AI models were developed, one including and one excluding pets as reservoir. The overall proportion of human infections attributed to a given reservoir was calculated as the sum of its *Pr* over cases divided by the total number of cases.

Differences in *Pr* for pets (Pr_p) were tested for the variables age, sex, urbanization degree, season, and pet ownership using the Kruskal-Wallis or the Mann-Whitney tests, as appropriate (alpha: 0.05).

Table 1. Reference *Campylobacter* strains used to feed the asymmetric island model for source attribution

Country	Chicken	Cattle	Sheep	Pig	Pets*	Environment	Reference
The Netherlands	236	0	9	0	13	106 (Water)	Data†
United Kingdom	73	46	46	72	5	50 (Sand)	Dingle *et al*. [12]
Scotland	239	90	90	88	15	133 (Wild birds)	Strachan *et al*. [17]
Switzerland	77	23	23	0	100	0	Korczak *et al*. [22]
Total	625	168	168	160	133	289	

*Include isolates from dogs and cats.
†Provided by the Central Veterinary Institute (CVI) in Lelystad, the Netherlands.

2.3. Risk factor analysis

Risk factor analysis was restricted to human campylobacteriosis caused by STs of probable pet origin and to human campylobacteriosis as a whole (all 737 typed cases, non-specific to probable pet origin). Risk factors for campylobacteriosis as a whole and for campylobacteriosis caused by STs attributable to reservoirs other than pets have been reported previously [23].

STs were assigned to pets based on their Pr_p. This was made the same way as previous studies [23–25]: the Pr_p distribution was assessed and a cut-off was determined to optimise the number of cases assigned to pets and the confidence as to their correct assignment derived by the highest possible Pr_p. A logistic regression analysis was then conducted to investigate risk factors for human campylobacteriosis caused by STs with at least 60% probability (cut-off $Pr_p \geq 0.60$) of originating from pets. This cut-off Pr_p resulted in the selection of 76 cases with a median Pr_p of 0.62 (mean Pr_p = 0.65, IQR = 0.08, min-max: 0.60-0.74) and represented the best trade-off between the increasing Pr_p (i.e. increase in pet specificity) and the decreasing number of cases includable in the analysis (i.e. decrease in statistical power and failure of the model to converge).

For preliminary significance testing, we assessed the association of 131 risk factors using a single-variable logistic regression analysis: variables with $p \leq 0.10$ were selected for inclusion in a multivariable logistic regression model. A backward stepwise selection procedure was applied and variables with $p<0.05$ were retained in the final model. The frequency matched variables and the level of education [18] were always included as covariates. Multivariable odds ratios (ORs) and corresponding 95% confidence intervals (CI) of risk factors for pet-associated human campylobacteriosis were presented together with those of a multivariable model built in the same fashion including all 737 typed cases that has been reported previously [23] and is presented here in abbreviated format to facilitate comparison.

The effect of the assignment cut-off Pr_p was checked by sensitivity analysis by repeating the analysis for a range of cut-off Pr_p, looking for significant changes in the risk factors in the reduced model. The low number of cases did not allow for the construction of models based on cut-off Pr_p larger than 0.60. Overall model significance and goodness-of-fit were verified by likelihood ratio chi-square and Hosmer-Lemeshow tests, respectively. Statistical analysis was performed using STATA 11.2.

3. RESULTS

3.1. Multilocus sequence types

Isolates from the 737 typed human cases were assigned to 154 STs (Figure 1). Of the 687 pet

faecal samples examined, 132 (19%) tested positive for *Campylobacter* spp. *Campylobacter* prevalence was 18% in samples specified of either dog or cat origin and 23% in the 263 samples of unspecified origin. A total of 499 isolates were recovered from the 132 positive samples (3.8 isolates per sample). Only 248 isolates had their origin specified (205 from dogs and 43 from cats).

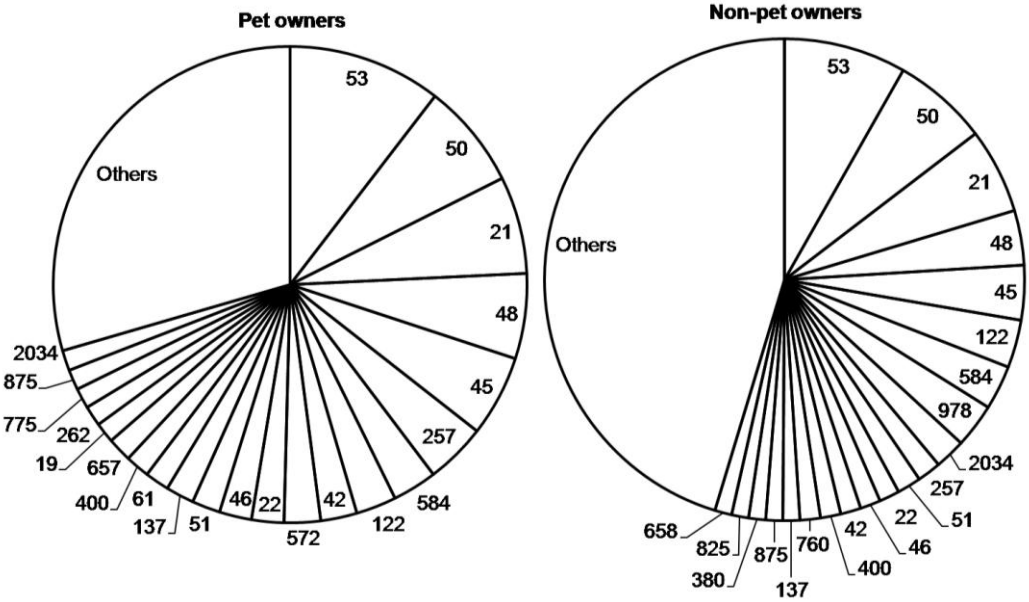

Figure 1. Sequence types identified among 737 *Campylobacter jejuni/coli* strains from human cases, subdivided by pet (dog and cat) ownership. Category 'others' includes sequence types that occurred fewer than five times.

Overall, 320 isolates (64%) were *C. upsaliensis* and *C. helveticus*, 100 (20%) *C. jejuni*, 33 (7%) *C. hyointestinalis* and *C. fetus*, 10 (2%) *C. lari*, 4 (1%) *C. coli*, and 32 (6%) unidentified. The 205 isolates from the dogs were: 158 (77%) *C. upsaliensis* and *C. helveticus*, 19 (9%) *C. jejuni*, 25 (12%) *C. hyointestinalis* and *C. fetus*, and 3 (2%) unidentified. The 43 strains from the cats were: 21 (49%) *C. upsaliensis* and *C. helveticus*, 21 (49%) *C. jejuni*, and 1 (2%) unidentified. The 104 *C. jejuni* and *C. coli* isolates from pets belonged to 49 STs: ST-45, ST-403, and ST-22 were the most represented (>25%, Figure 2); ST-403 was the most represented in the dogs (4 strains out of 19) and ST-45 in the cats (4 strains out of 21).

The PSI for STs of pet owners and non-pet owners (Figure 1) was 0.69 (95% CI 0.50–0.88). PSI for STs of pets and pet owners was 0.39 (95% CI 0.27–0.51) and that for pets and non-pet owners was 0.33 (0.20–0.45). PSI for pets and dog owners was 0.43 (95% CI 0.26–0.60) and that for pets and puppy owners was 0.51 (95% CI 0.30–0.64).

In 68 cases, isolates were typed from both pets and patients living in the same households. Of these, two owners (2.94%) were infected with the same ST found in their pets. These were a 44-year-old man and his dog infected with ST-45 and a 47-year-old man and his pet infected with ST-658. Given our ST distributions in humans and pets and the overall prevalence of *C. jejuni* and *C. coli* in pets, the expected probability of finding an identical ST in humans and pets in a one-to-one relationship purely by random chance was 0.198%, which corresponds to an expected 0.134 cases out of 68. The difference between the observed (2) and expected (<<1) co-isolation of identical STs in humans and pets was statistically significant (binomial probability test, p = 0.008).

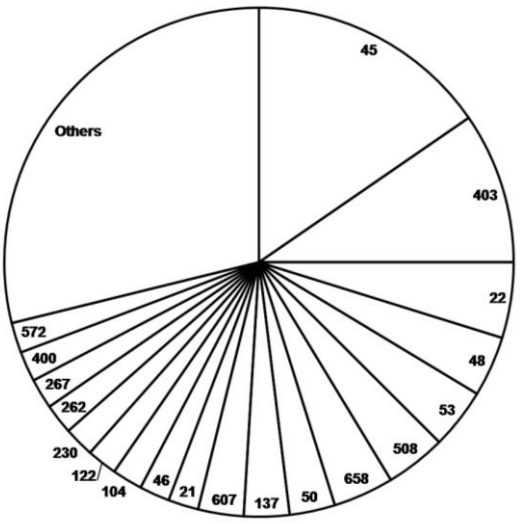

Figure 2. Sequence types identified among 104 *Campylobacter jejuni/coli* strains from dogs and cats owned by people with *Campylobacter jejuni/coli* infections. Category 'others' includes sequence types that occurred once.

3.2. Attribution of human infections

The AI model without pets (Figure 3) estimated that 77% (95% CI 75–79%) of human infections originated from chicken, followed by cattle 18% (95% CI 16–19%), environment 2% (95% CI 1–3%), sheep 2% (95% CI 1–3%), and pigs 1% (95% CI 0.1–1%). When pets were also included (Figure 3), cases were attributed as follows: chicken 63% (95% CI 61–65%); pets 25% (95% CI 23–26%); cattle 11% (95% CI 9–11%); pigs 0.5% (95% CI 0.1–0.8%); sheep 0.4% (95% CI 0.3–0.5%); and environment 0.1% (95% CI 0.1–0.2%).

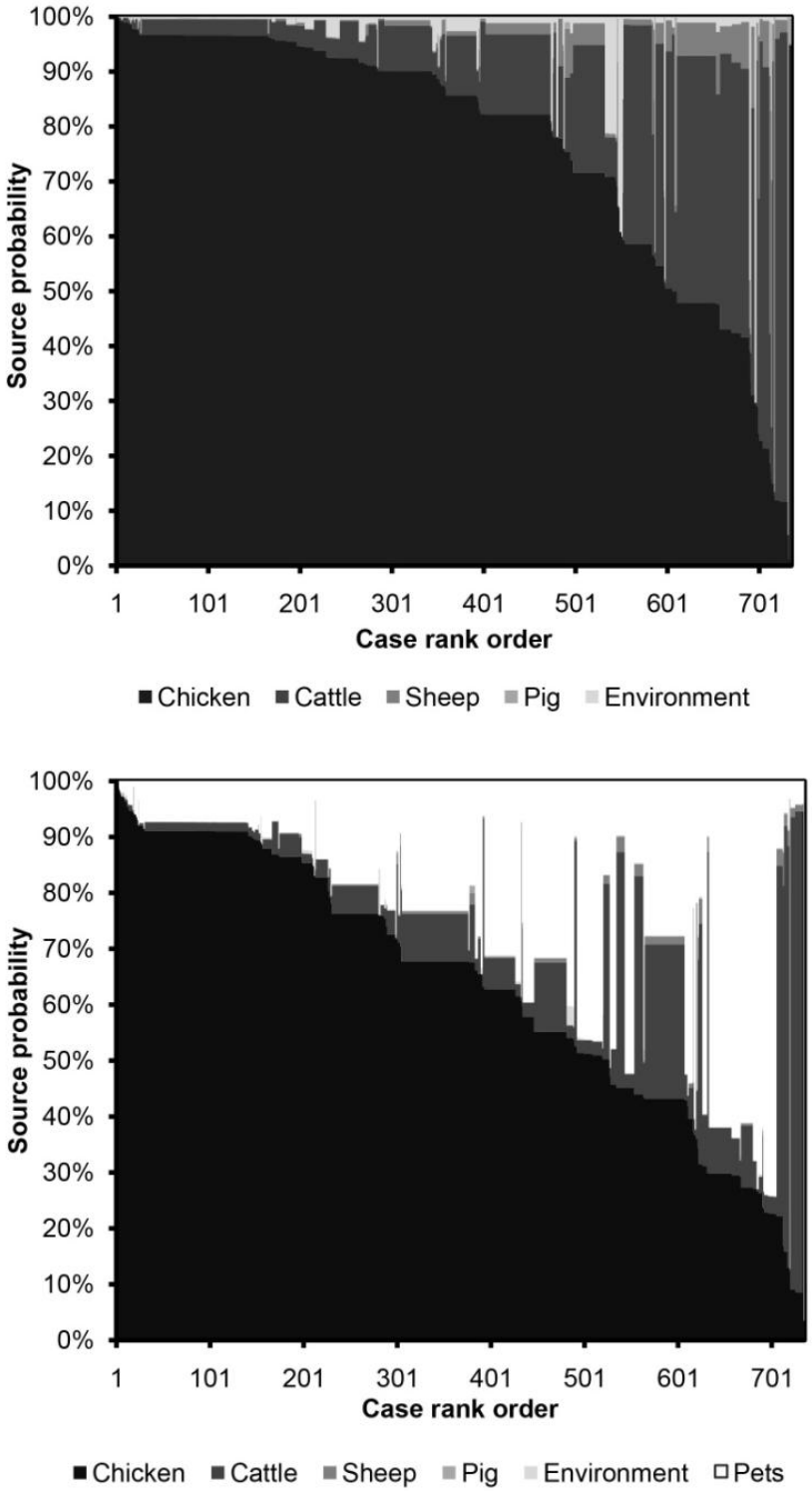

Figure 3. Assignment source probability (%) estimated by the asymmetric island model with and without pets represented as a matrix plot. Each human case is a vertical column colored according to the probability it came from each considered source. To aid visualization, cases are ordered horizontally according to the probability attributed to chicken.

STs predominantly associated with pets included in the risk factor analysis ($Pr_p \geq 0.60$) were: ST-403 ($Pr_p = 0.76$), ST-508 (0.74), ST-586 (0.74), ST-1326 (0.74), ST-878 (0.73), ST-3130 (0.72), ST-1911 (0.70), ST-2088 (0.67), ST-47 (0.67), ST-657 (0.63), ST-2151 (0.62), ST-2130 (0.61), ST-122 (0.61), ST-22 (0.61), and ST-677 (0.60).

The average Pr_p was significantly higher ($p = 0.028$) in dog owners (median $Pr_p = 0.25$, IQR = 0.3, $n = 221$) compared to non-dog owners (median $Pr_p = 0.18$, IQR = 0.23, $n = 516$), and this difference became more evident when considering those owning a puppy (median $Pr_p = 0.31$, IQR = 0.43, $n = 35$, $p = 0.001$) or those owning both puppies and adult dogs (median $Pr_p = 0.49$, IQR = 0.34, $n = 12$, $p = 0.003$).

3.3. Risk factors for pet-associated human campylobacteriosis

Several factors were significantly associated with *C. jejuni* and *C. coli* infection in the overall model (Table 2), including factors concerning contact with pets, such as ownership of several adult dogs and at least one puppy (OR 2.5, 95% CI 1.1–5.8); ownership of one or more cats (OR 1.4, 95% CI 1.2–1.8); and contact with dogs outside the household (OR 0.6, 95% CI 0.5–0.8). However, only three risk factors (two of which related to contact with pets) were significant in the model for campylobacteriosis of probable pet origin (Table 2). These were: 1) ownership of a puppy (OR 3.7, 95% CI 1.4–10.0); 2) ownership of several adult dogs and at least one puppy (OR 9.2, 95% CI 2.7–32.0); and 3) recent use of proton-pump inhibitors (OR 11.1, 95% CI 5.4–22.9). The first risk factor was unidentified in the overall model. No significant interactions of these risk factors with age, gender, urbanization degree, season, or level of education were found.

4. DISCUSSION

This study shows that there is a high degree of overlap between human and pet *C. jejuni* and *C. coli* STs and that dog owners, especially puppy owners, are at higher risk for infection with STs associated with pets than controls and non-dog owners. Furthermore, there were two cases out of 68 where a patient and a pet living in the same household were infected with an identical ST. Although this may seem a minor proportion, it is considerable if we reflect on both the adopted sampling scheme (particularly the relatively long time lag between human and pet sampling) and the low occurrence of severe, symptomatic campylobacteriosis usually detectable by passive surveillance. Moreover, *C. jejuni* carriage in pets is infrequent and of short-term [26]. Accordingly, the expected occurrence of identical STs in a one-to-one relationship was significantly lower than that observed. Furthermore, although the 95% CIs of the PSI were largely overlapping, the point estimates showed a trend towards similarity of pet and human ST frequency distributions according to pet ownership. Taken together these results suggest that dog ownership increases significantly the risk of acquiring *C. jejuni* and *C. coli* infection caused by STs originating from pet and that co-isolation of identical strains in humans and their pets occurs significantly more often than expected by chance.

There are four possible scenarios arising from our findings: 1) humans and pets become infected from the same source; 2) humans and pets become infected from different sources that incidentally carry the same strain; 3) humans become infected from dogs; 4) dogs become infected from humans. While the second scenario seems unlikely because of the large variety of existing STs, there may be many common sources of infection for pets and humans [27]. This is mainly because pet foods and treats contain ingredients of animal origin. Moreover, pets are increasingly regarded as real family members and tend to be fed with the same foods as their owners [28]. Feeding of a homemade diet or table and kitchen food scraps, especially raw meats, offal, and bones, is a risk factor for *Campylobacter* carriage in pets [29-31], and pets carrying *Campylobacter* may eventually act as reservoirs for transmission to humans either directly or by contaminating the household and immediate environments [27, 28]. As the sampling design was non-directional in the transmission of infection, our results support evidence for genetic association of *C. jejuni* and *C. coli* strains between humans and their pets but do not prove that transmission of such strains occurs from pets to humans or vice versa.

Table 2. Risk factors for human *Campylobacter jejuni/coli* infection in general (overall model) and for infection caused by *C. jejuni/coli* strains of pet origin (pet model) as assigned by the asymmetric island model for source attribution.

Risk factor (% imputed missing values*)	OR (95% CI) Overall model from Mughini Gras *et al*. [23]†‡	OR (95% CI) Pet model†§
Food consumption		
Chicken (1)	**1.5 (1.2–1.9)**	ns
Undercooked meat (5)	**2.1 (1.6–2.7)**	ns
Meat cooked at a barbecue, grill, or microwave oven (5)		
in urban areas	**1.2 (0.7–2.2)**	ns
in urbanized areas	**1.7 (1.3–2.2)**	ns
in rural areas	**3.0 (1.8–4.9)**	ns
Eating in a restaurant (0)	**1.3 (1.1–1.6)**	ns
Meat in paste (croquette, sausage roll, pastry) (5)	0.8 (0.6–1.0)	ns
Pasteurized milk (1)	0.8 (0.6–0.9)	ns
Pasteurized dairy products other than milk or cheese (2)	0.6 (0.5–0.7)	ns
Salad (2)	0.7 (0.6–0.9)	ns
Fruit with peel (2)	0.7 (0.6–0.8)	ns
Chocolate (2)	0.6 (0.5–0.7)	ns
Nuts (3)	0.6 (0.5–0.8)	ns
Seafood (4)	0.5 (0.4–0.7)	ns
Vegetarian diet (0)	0.4 (0.2–0.9)	ns
Contact with animals		
Contact with dogs owned by other people (3)	0.6 (0.5–0.8)	ns
Ownership of one dog aged <1 year (0)	ns	**3.7 (1.4–10.0)**
Ownership of more than one dog, at least one dog aged <1 year (0)	**2.5 (1.1–5.8)**	**9.2 (2.7–32.0)**
Ownership of one or more cats (1)	**1.4 (1.2–1.8)**	ns
Recent use of medications		
Antibiotics (0)	0.4 (0.2–0.8)	ns
Proton–pump inhibitors (0)	**3.7 (2.5–5.5)**	**11.1 (5.4–22.9)**
Kitchen hygiene and food processing		
Not cleaning a knife when using it for raw meat and other foods (1)	**1.7 (1.1–2.6)**	ns
Washing hands before food preparation (0)	0.6 (0.4–0.9)	ns
Contact with people with gastroenteritis outside the household (3)	**1.5 (1.1–2.1**	ns
Having a gastrointestinal chronic disease (0)	**2.4 (1.8–3.2)**	ns

OR = odds ratio; 95% CI = 95% confidence intervals; ns = not significant (p>0.05). **Boldface** indicates the risk factors; protective factors are in normal font.
*% of imputed missing values in the whole dataset.
†Adjusted for age, sex, degree of urbanization, season, and level of education.
‡*n* = 3856 (737 cases and 3119 controls).
§*n* = 3195 (76 cases and 3119 controls).

The contribution of pet ownership to human infections, as derived from case-control studies, appears to not exceed 10% [7]; thus, the pet attribution found in this study (25%) seems to be overestimated. Presumably, this is an artefact of the attribution process, as pets are not the main reservoir of STs found in pets and humans. The AI model could therefore have attributed many cases to the pets themselves instead of the common reservoirs. When including pets in the AI model, cases attributable to chicken decreased measurably (–14%), followed by cattle (–7%), sheep (–2%), and the environment (–2%). The extent of this decrease is suggestive of the reservoirs from which pets may acquire infection in parallel with humans. Overall, figures provided by the AI model without pets concur with those of similar studies [13–7].

We found that ownership of dogs, particularly puppies and several adult dogs, became a predominant risk factor when considering the cases with a high Pr_p. *Campylobacter* prevalence is higher in puppies and adult dogs housed in groups, possibly due to low levels of acquired immunity and dog to dog transmission, respectively [6,32]. Moreover, while puppies are usually housed indoors and have closer contacts with their owners, adult dogs living in groups are likely to have (unsupervised) outdoor access and act as vectors for environmental strains [23], especially if they have access to fields grazed by livestock or wildlife [27]. Eventually, owners walking their dogs may be particularly exposed to such environmental strains. While dog ownership increases the risk for pet-associated infections, contacting dogs outside the household appears to be protective. A possible explanation is that contacting dogs other than their own encourages individuals to undertake protective actions such as hand washing [23].

In this study, cat ownership, unlike dog ownership, was not a significant risk factor for the most pet-associated STs. This is in accordance with canine behaviour that generally results in frequent soil and water contact, whereas cats usually hide and bury their faeces and lick their fur intensively,

making it easier to remove any possible *Campylobacter*-contaminated matter [4].

It has been shown that individuals acquiring *C. jejuni* and *C. coli* infection from different reservoirs have different associated risk factors [23, 24]. Modelling of MLST data is useful for determining where infection is likely to be acquired from. However, most cases cannot be attributed to a single reservoir; only those cases infected with rare STs, i.e. occurring 1-5 times on average [23], usually show a high *Pr* (i.e. ≥0.5) for a given reservoir and possess unique risk factors associated with that reservoir. Also in this study, ST's reservoir specificity and rareness seem to correlate, as the Pr_p-based selection led to 76 cases belonging to 15 different STs (5 cases per ST). Common STs are difficult to disentangle and assign to specific reservoirs because they could potentially originate from several reservoirs simultaneously. Moreover, the high *Pr* for a given reservoir cannot be excluded to vary over time: new STs may emerge at low frequencies in specific reservoirs (i.e. high *Pr* for a reservoir) and then either disappear or become increasingly widespread (i.e. almost equal *Pr* for different reservoirs).

ST-45 and ST-658 were co-isolated in pets and their owners. Pets corresponded to the first and second most likely reservoir for ST-658 (Pr_p = 0.59) and ST-45 (0.31), respectively. The globally widespread ST-45 usually predominates in chicken [12,15,17,33–35], as also indicated by our AI model with pets (*Pr* for chicken = 0.62). However, in our study and in another one from the UK [27], ST-45 was the most frequent ST in pets and was associated with contact with pets in Finland [33], suggesting that its circulation among pets is extensive. Furthermore, as ST-45 was overrepresented in surface water [36], and open drains/pools have been associated with *Campylobacter* carriage in dogs [29], it has been hypothesized that ST-45 is an environmentally adapted ST that mainly infects humans through transmission pathways other than food, including contact with pets [30, 36].

In this study, the overall proportion of *Campylobacter*-positive samples in pets (19%) was relatively low compared to previous figures from the Netherlands (77%) based upon 22 healthy and 8 diarrheic household dogs aged 3 months-14 years [37]. In other studies [9,29–31], *Campylobacter* prevalence ranged between 15% and 76% for dogs, and 11% and 43% for cats, but can be as high as 87% and 75% in kennelled dogs and cats, respectively [32]. This variability is due to differences in age, sex, breed, diet, and housing of the examined animals, but also to the different management of faecal samples. The delivery of pet samples by mail could have affected the survival of the most fragile strains, possibly skewing our sample towards the most resistant ones.

We also found a relatively large proportion of *C. upsaliensis* and *C. helveticus* (64%). In a Danish longitudinal study in which 366 faecal samples from 26 household dogs were also sent by mail [26], 76% were *Campylobacter*-positive, 75% of which were *C. upsaliensis* and 19% *C. jejuni*. The Danish study included only puppies tested monthly until two years of age, a factor that may explain the higher overall isolation rate. However, the dog and cat *Campylobacter* species distribution we found is comparable with other reports [29–31,37], suggesting that taking delivery of pet samples by mail did not bias the *Campylobacter* species distribution.

In conclusion, we compared *C. jejuni* and *C. coli* strains from pets and their owners using MLST and investigated risk factors for campylobacteriosis of probable pet origin by combining source attribution and case-control data. Although pets and humans share many sources of infection and directionality of transmission between humans and pets could not be inferred, the combined analysis and the co-isolation of identical STs in pets and their owners suggest that dog, and particularly puppy, ownership is a risk factor for *C. jejuni* and *C. coli* infection caused by STs of probable pet origin and that co-isolation of identical strains in humans and their pets occurs more frequently than expected. Probably, this evidence could have been even stronger if the time lag between the two samples had been shorter.

Attributing human infections to pets may be deceptive when the goal is to identify the original reservoirs, as pets may artificially account for an abnormal amount of cases because they are, like humans, predominantly "final" hosts for *C. jejuni* and *C. coli*. Conversely, the contribution of the other reservoirs will be underestimated and probably biased towards those reservoirs from which pets acquire infection.

It is unclear to what extent the increased risk of pet-associated campylobacteriosis in dog owners we found is

an indication of other unmeasured factors, such as owner's personality traits, lifestyle, income, disability or other health problems, which can plausibly influence the chance of becoming infected and the decision and manner of owning a pet. The zoonotic risk posed by pets should therefore be put into context, depending on factors such as level of *Campylobacter* carriage and intensity and type of contact between pets and humans.

Besides the previously identified risk factors for *C. jejuni* and *C. coli* infection [7], there are different risk factors depending upon the attributable reservoir [23]. In this study, we explored risk factors for infection with STs attributable to pets, a poorly characterized reservoir of human campylobacteriosis. This analysis provided insight into reservoir-specific risk factors and transmission pathways for human campylobacteriosis, allowing for a better characterization of the zoonotic risk posed by pets. By enhancing our ability to characterize this zoonotic risk, public health initiatives can be better informed.

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Chapter 8

Campylobacteriosis in returning travelers and potential secondary transmission of exotic strains

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Campylobacteriosis in returning travelers and potential secondary transmission of exotic strains

ABSTRACT

Multilocus sequence types (STs) were determined for 232 and 737 *Campylobacter jejuni*/*coli* isolates from Dutch travelers and domestically-acquired cases, respectively. Risk factors for travel-related campylobacteriosis, and for domestically-acquired campylobacteriosis caused by exotically-introduced STs (carried by the returned travelers), were investigated. Traveling to Asia, Africa, Latin America and the Caribbean, and Southern Europe increased significantly the risk of acquiring campylobacteriosis compared to traveling within Western Europe. Besides eating chicken, using antacids, and having chronic enteropathies, we identified eating vegetable salad outside Europe, drinking bottled water in high-risk destinations, and contacting raw pork as specific risk factors for travel-related campylobacteriosis. Risk factors for domestically-acquired campylobacteriosis caused by exotic STs involved predominantly person-to-person contacts around popular holiday periods. We concluded that risk factors for travel-related campylobacteriosis differ from those for domestically-acquired infections and that returning travelers may carry several exotic strains that might subsequently spread to domestic populations.

1. INTRODUCTION

Diarrheal infections remain a major concern for travelers, especially for those bound for destinations where relatively substandard hygienic conditions exist. A Dutch study showed that in a sample of 1202 individuals traveling to developing countries, 50% experienced ≥1 diarrheal episodes [1].

Campylobacteriosis is a leading cause of traveler's diarrhea, particularly in travelers returning from Southern and South-Eastern Asia [2,3]. In The Netherlands (16.5 million population), an estimated 90,000 campylobacteriosis cases occur annually, with ~12% of them being estimated to be travel-related [4]. Moreover, fluoroquinolone-resistant *Campylobacter* infections occur significantly more often in travel-related cases [3,5].

Travelers are particularly prone to experiencing (symptomatic) *Campylobacter* infections [2,6,7]. Among others, susceptibility to disease is associated with duration of foreign stay. For instance, duration of residence of expatriates in Nepal was linearly correlated with protection from diarrheal infection [8], and travelers experiencing multiple diarrheal episodes had a shorter duration of symptoms after the first episode [1]. Similarly, among US expatriates in Thailand, campylobacteriosis occurred significantly more often in those living there for <1 year [9]. A documented instance of acquired immunity in developed countries is with people professionally exposed. Newly employed poultry abattoir workers in Sweden have shown to suffer more often from campylobacteriosis than their longer employed colleagues [10]. If partial immunity to (severe) disease is acquired over time with repeated exposure, then such protection should correlate with age. Indeed, campylobacteriosis incidence peaks in infancy worldwide and older age groups are significantly less prone to infection with common *Campylobacter* strains compared to the young [11]. Moreover, Swedish travelers going to countries such as Germany, France, Belgium, The Netherlands, Austria, Luxemburg or Switzerland, all of which are developed countries with high hygienic standards, still have a 4.4–21 times higher risk of acquiring campylobacteriosis compared to those traveling to neighboring Norway [12]. A study comparing *Campylobacter* multilocus sequence typing (MLST) datasets from different countries has further highlighted the importance of geographical distance in strain

dissimilarity [13]. Moreover, serological studies of patients and volunteers infected with campylobacters (reviewed by [14]) have revealed an array of immunogenic components and elicited antibodies displayed little cross-reactivity, indicating considerable antigenic variation. Although core-genome (as described by MLST) is not necessarily related to antigens, and cross-protection is expected to develop for strains sharing similar antigenic properties, the higher risk of campylobacteriosis in travelers does not seem to be limited to higher levels of exposure in developing countries, but also to the possible presence of "new" *Campylobacter* strains, endemic in the different regions, to which travelers have hardly been exposed before [14]. It follows that, probabilistically, these "new" strains are more likely to be associated with regionally untested antigens than widespread strains, and acquired protection may be ineffective when exposed to uncommon strains, as evidenced by a Canadian study [15].

It is conceivable that the infected, but not necessarily symptomatic [14], returning travelers, may introduce into the domestic population several "exotic" strains with a higher probability of possessing antigens that are underrepresented in the local reservoirs, i.e. food-producing animals, pets and wildlife. These exotic strains may therefore constitute a distinctive, primarily human-restricted *Campylobacter* population that may have at least the potential to spread domestically via the person-to-person pathway. Although *Campylobacter* person-to-person transmission is believed to be uncommon and up to 66% and 21% of laboratory-confirmed cases in The Netherlands are attributable to chicken and cattle, respectively [16], it has been shown that campylobacteriosis household outbreaks are more common than believed [17] and up to 18% of putatively household outbreak-related cases are suggestive of secondary spread [18]. This raises the question to what extent exotically-introduced strains may spill-over into the domestic population and at first spread anthroponotically.

Herewith we investigated the MLST profiles of *Campylobacter* strains isolated from travelers returned to The Netherlands in comparison with those from domestically-acquired cases. We also investigated risk factors for travel-related campylobacteriosis by comparing the exposures of the returned travelers with those of travelers in the control population. Furthermore, we used a population genetics model for source attribution to estimate the probability that the domestically-acquired infections were caused by exotic strains, putatively carried by the returned travelers. Finally, risk factors for exotically-introduced domestic campylobacteriosis were investigated.

2. METHODS

2.1. Data

An earlier case-control study on risk factors for campylobacteriosis conducted in The Netherlands between April 2002 and March 2003 [6] formed the basis of this study. Isolates of 3115 *Campylobacter jejuni*/*coli* cases identified by the Dutch Regional Public Health Laboratories (RPHL) through passive surveillance were sent to the Dutch Central Veterinary Institute (CVI) for molecular speciation [19,20]. Controls were selected from RPHL population registries by frequency matching (aiming at two per case) according to age, sex, urbanization degree and season [6,16,21]. Cases and controls were asked to fill in a questionnaire regarding foreign travel, food consumption, kitchen hygiene, contact with animals, contact with gastroenteritis cases, occupation, recreational activities, medication use and chronic disease history. Questions covered the seven days prior to symptoms onset (cases) or questionnaire completion (controls). Missing values were handled using multiple imputation [6,21].

Cases/controls not returning the questionnaire and/or living abroad were excluded, leaving 1428 cases and 3363 controls enrolled in the study. Of these, 328 cases and 244 controls had traveled abroad with ≥1 overnight stay in the destination country. A total of 66 countries were visited, with 36 cases and 27 controls visiting >1 country during the same travel. For three cases and four controls the travel destination was unknown. Destination countries were grouped into travel regions by adapting the United Nations geoscheme (http://unstats.un.org/unsd/methods/m49/m49regin.htm#europe, Table 1).

Isolates from 737 non-travelers (domestically-acquired cases) and 232 travelers were typed using MLST [22]. Association of the travelers' five most frequent sequence types (STs) and clonal complexes

(CCs) with travel regions was tested using χ^2 or Fisher exact tests. Proportional Similarity Index (PSI) [13] was used to measure the (dis)similarity between ST frequency distributions of travelers and non-travelers. PSI ranges between 0 (no common ST) and 1 (identical distributions). Simpson's index of diversity was calculated to define the ST diversity of travelers and non-travelers as the probability that two randomly selected individuals were infected with different STs [23].

Table 1. Region of destination and length of stay for cases and controls that had traveled abroad.

Destination region*	Cases (*n* = 328)		Controls (*n* = 244)	
	Exposed (%)	Days stayed†	Exposed (%)	Days stayed†
Northern Europe‡	3.3	4 (4–6)	8.6	13 (4–28)
Western Europe§	22.5	9 (4–16)	59.0	12 (4–19)
Eastern Europe¶	4.2	11 (6–21)	4.9	15 (4–22)
Southern Europe#	22.5	14 (8–16)	24.1	11 (7–16)
Northern Africa**	8.5	14 (8–26)	1.2	27 (16–31)
Sub–Saharan Africa††	4.2	21 (15–57)	0.4	3 (3–3)
Western Asia‡‡	13.7	14 (7–15)	2.0	14 (7–14)
South–East Asia and China§§	16.1	20 (15–27)	0.8	11 (9–13)
Southern Asia¶¶	3.6	38 (18–49)	0.4	22 (22–22)
Oceania##	0.6	78 (34–122)	0.4	28 (22–22)
North America***	None	None	0.8	60 (11–109)
Latin America and the Caribbean†††	4.8	22 (15–55)	0.4	7 (7–7)
Unknown	0.9	11 (11–14)	1.6	11 (10–11)

*Adapted from the United Nations scheme of the composition of macro geographical (continental) regions, geographical sub-regions, and selected economic and other groupings (http://unstats.un.org/unsd/methods/m49/m49regin.htm#europe). †Median (25th-75th percentile). ‡Includes travelers returning from the UK, Ireland, Denmark, Sweden, Norway, and Finland. §Includes travelers returning from Germany, France, Belgium, Austria, Luxemburg, and Switzerland. ¶Includes travelers returning from the Czech Republic, Hungary, Poland, Slovakia, and Romania. #Includes travelers returning from Spain, Italy, Portugal, Greece, Croatia, and Malta. **Includes travelers returning from Morocco, Egypt, and Tunisia. ††Includes travelers returning from Benin, Cameroon, Ethiopia, Ghana, Kenya, Mali, Nigeria, Rwanda, Tanzania, Botswana, Burkina Faso, South Africa and Namibia. ‡‡Includes travelers returning from Turkey, Jordan and Iraq. §§Includes travelers returning from China, Indonesia, Malaysia, Singapore, Philippines, Thailand, and Vietnam. ¶¶Includes travelers returning from India, Nepal, and Bangladesh. ##Includes travelers returning from Australia and Fiji Islands. ***Includes travelers returning from the USA. †††Includes travelers returning from Bolivia, Ecuador, Peru, Venezuela, Chile, Costa Rica, Cuba, Dominican Republic, Haiti, Mexico, and Guatemala.

2.2. Source Attribution

The Asymmetric Island (AI) model, a Bayesian population genetics algorithm for modeling *Campylobacter* evolution and transmission [24], was used to estimate the probability (*Pr*) for the 737 non-travelers to be infected with exotically-introduced STs or with STs originating from four putative animal reservoirs (chicken, cattle, sheep, pig) or from the environment (water, sand, wild-birds), a proxy for other unidentified reservoirs putatively of wildlife origin [16]. This study was restricted to campylobacteriosis of probable exotic origin. Results regarding the other animal and environmental sources have been reported elsewhere [13,16].

To run the AI model, *C. jejuni*/*coli* MLST data from the aforementioned animal and environmental sources were supplied by the CVI and supplemented with other data [22,25,26] to provide a representative dataset for each source (Table 2). Supplementary data were selected among other published datasets (reported in [13]) using Smid's methodology, which allows for the selection of non-local and non-recent MLST datasets for *Campylobacter* source attribution while minimizing potential biases [13,16]. Differences in *Pr* for exotic origin (Pr_e) were tested for the variables age, sex, and season using the Kruskal-Wallis or Mann-Whitney tests.

2.3. Risk Factors for Travel-related Campylobacteriosis

Logistic regression was used to investigate risk factors for travel-related campylobacteriosis. The 325 diseased travelers and the 238 healthy travelers with known travel destination were included as cases and controls, respectively. Analysis was performed in the same way as in previous studies [6,16,21]. Factors showing a $p \leq 0.10$ for the association with the outcome in the single-variable analysis were selected for inclusion in a multivariable model. A backward stepwise procedure was applied and variables with a $p < 0.05$ were retained in the final model. Education level [16], travel region, and days stayed were always included as covariates to control for confounding in addition to the frequency-matched variables. As travel regions were almost mutually exclusive, Western

Europe, which The Netherlands belongs to, was made the base category against which the other regions were assessed.

To explore if the risk factors of the reduced model differed according to age, sex, education level, season, and travel region, we also tested for their two-way interactions. The final multivariable model was then expanded to include significant interactions. Overall model significance and goodness-of-fit were verified by likelihood ratio χ^2 and Hosmer-Lemeshow tests, respectively. The best-fitting model was identified using the Akaike Information Criterion (AIC). Bias-corrected bootstrap confidence intervals were also calculated (1000 iterations) and compared with the standard ones. As these did not differ significantly, the standard ones were reported. Statistical analysis was performed using STATA 11.2.

Table 2. *Campylobacter jejuni/coli* strains typed with MLST used to feed the asymmetric island model for source attribution.

Country	Humans (travelers)	Chicken	Cattle	Sheep	Pig	Environment	Reference
The Netherlands	232*	236	0	9	0	106 (Water)	Data†
United Kingdom	0	73	46	46	72	50 (Sand)	[22]
Scotland	0	239	90	90	88	133 (Wild birds)	[25]
Switzerland	0	77	23	23	0	0	[26]
Total	232	625	168	168	160	289	

*From the *Campybobacter jejuni/coli*-diseased travelers of the CaSa study [6].
†Provided by the Central Veterinary Institute (CVI) in Lelystad, The Netherland.

2.4. Risk Factors for Exotically-introduced Domestic Campylobacteriosis

To investigate risk factors for domestically-acquired campylobacteriosis caused by STs of probable exotic origin, MLST data of travelers were included as an additional source in the AI model. Similar to previous studies [16,27,28], the Pr_e distribution was assessed and a cut-off was determined to optimize the number of domestic cases assigned to be exotically-introduced and the confidence as to their correct assignment derived by the highest possible Pr_e. Logistic regression was then used to investigate risk factors for domestically-acquired campylobacteriosis caused by STs with at least 77% probability (cut-off $Pr_e \geq 0.77$) of originating from abroad. This cut-off Pr_e resulted in the selection of 77 cases with a median Pr_e of 0.89 (mean = 0.88, range: 0.77-0.99) belonging to 35 different STs. The 3119 non-traveling controls were included in this analysis.

The effect of the assignment cut-off Pr_e on the risk factors was checked by sensitivity analysis, repeating this for different cut-off Pr_e from 0.5 to 0.9. Low numbers of cases did not allow for the construction of models based on cut-off $Pr_e > 0.9$. Finally, a case-case analysis comparing exposures of domestic infections with exotic vs. non-exotic STs was performed.

3. RESULTS

3.1. Genotypes

The 737 typed strains from non-travelers were assigned to 154 STs and 28 CCs, whereas those from the 232 travelers were assigned to 127 STs and 23 CCs. Twenty-eight STs from non-travelers and 23 STs from travelers were unassigned to previously identified CCs.

In non-travelers, the top five STs (ST-53, ST-50, ST-21, ST-48, ST-45) accounted for >25% of cases and the top five CCs (CC-21, CC-45, CC-206, CC-257, CC-48) for >50% of cases (Figures 1 and 2). In travelers, the top five STs (ST-572, ST-21, ST-50, ST-53, ST-353) accounted for ~20% of cases and the top five CCs (CC-21, CC-353, CC-828, CC-206, CC-52) for >50% of cases (Figures 1 and 2). STs occurring once accounted for 46% and 70% of STs in non-travelers and travelers, respectively. PSI between travelers and non-travelers was 0.47 (95% confidence interval [CI]: 0.34–0.59) while Simpson's index was 0.972 (95% CI: 0.968–0.976) in non-travelers and 0.988 (0.984–0.992) in travelers.

There were 68 STs (#74 cases) found only in travelers and absent in any of the considered sources and in non-travelers (Table 3). Most cases (73%) infected with these traveler-only STs had traveled to Asia or Africa vs. 46% of all travel-related cases returning from these continents (z-test, $p<0.001$). Conversely, 23% of cases infected with traveler-only STs had traveled within Europe vs. 53% of all travel-related cases traveling within Europe (z-test, $p<0.001$).

ST-572 was significantly overrepresented (64%) in travelers from Western Europe (p = 0.001); ST-50 in those from Western Asia (40%; p = 0.014); ST-53 in those from Southern Europe (50%; p = 0.035); ST-353, CC-353 and CC-828 in those from Northern Africa (38%, 27%, and 46%; p = 0.003, p<0.001, and p<0.001, respectively); CC-52 in those from Eastern Europe (23%; p = 0.001).

The 35 STs with $Pr_e \geq 0.77$ included in the risk factor analysis for exotically-introduced domestic campylobacteriosis are reported in Figure 3. The five most exotic STs ($Pr_e \geq 0.98$) were ST-4284, ST-4278, ST-4311, ST-2123 and ST-3015. There was a significant seasonal effect (p = 0.036) on Pr_e, which peaked in October-December and decreased in April-June. No significant age and sex effects on Pr_e were found.

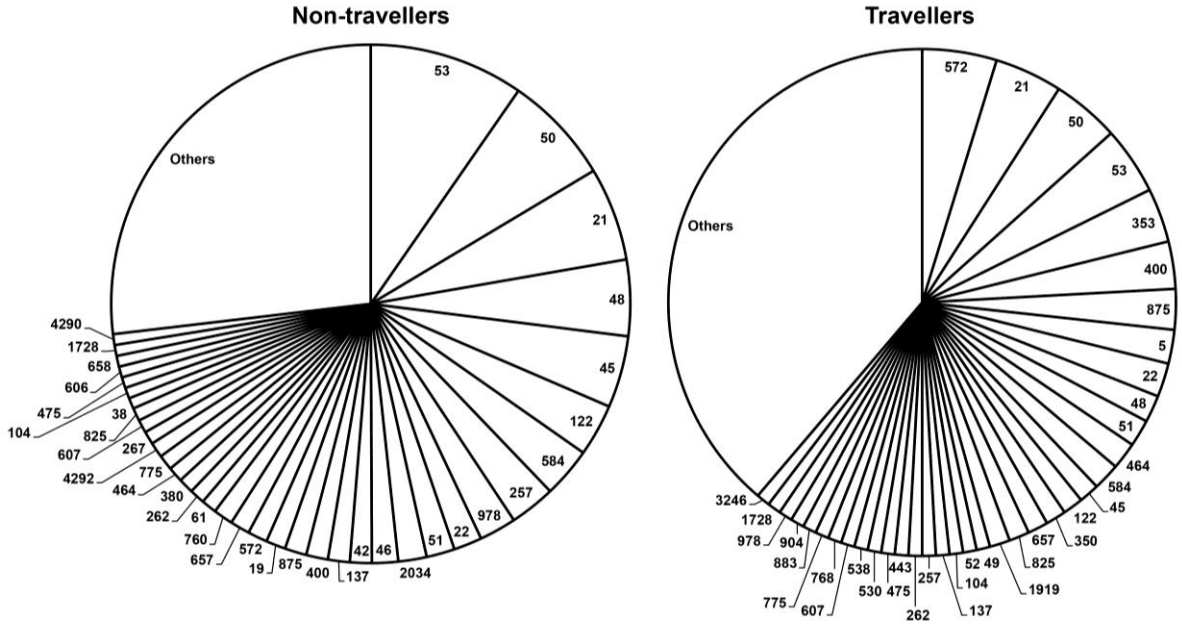

Figure 1. Sequence types identified amongst *Campylobacter jejuni*/*coli* strains isolated from the 737 non-travelers (infections acquired in the Netherlands) and the 232 travelers returning to The Netherlands. Category 'others' includes sequence types that occurred less than five (non-travelers) and two (travelers) times.

3.2. Risk Factors for Travel-related Campylobacteriosis

Compared to traveling within Western Europe, traveling to any region in Asia, Africa, Latin America and the Caribbean, and Southern Europe posed a higher risk to acquire campylobacteriosis (Table 4), whereas the risk posed by Northern and Eastern Europe and Oceania, as well as the length of stay, were not significant (p>0.05).

Significant risk factors for travel-related campylobacteriosis (Table 4) were: using proton-pump inhibitors, consuming vegetable salad when traveling outside Europe, contact with raw pork, having chronic enteropathies, drinking bottled water when traveling to Southern Europe or non-European countries, and consuming chicken. Consuming yoghurt and being employed in healthcare were protective.

3.3. Risk Factors for Exotically-introduced Domestic Campylobacteriosis

Risk factors for exotically-introduced domestic campylobacteriosis (Table 5) were: not washing hands after toilet visit in January-April 2003, using proton-pump inhibitors, being a school-going child in April–June 2002 and October–December 2002, attending public swimming pools in October–December 2002, and contact with gastroenteritis cases outside the household. Consuming yoghurt was protective. Sensitivity analysis of the cut-off Pr_e revealed that this had no effect on the main results. In case-case analysis, the same risk factors were identified apart from consuming yoghurt and antacids (no longer significant), and therefore no further results are presented.

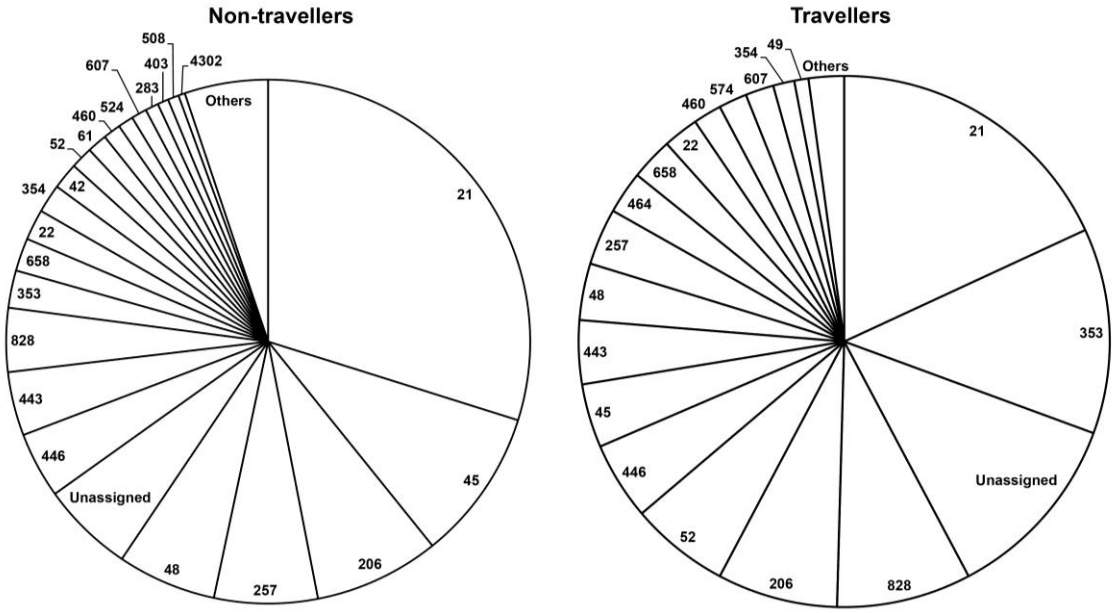

Figure 2. Clonal complexes assigned to 154 and 127 Campylobacter jejuni/coli sequence types identified from the 737 non-travelers (infections acquired in The Netherlands) and the 232 travelers returning to The Netherlands, respectively. Category 'others' includes clonal complexes that occurred less than twice (non-travelers) and once (travelers).

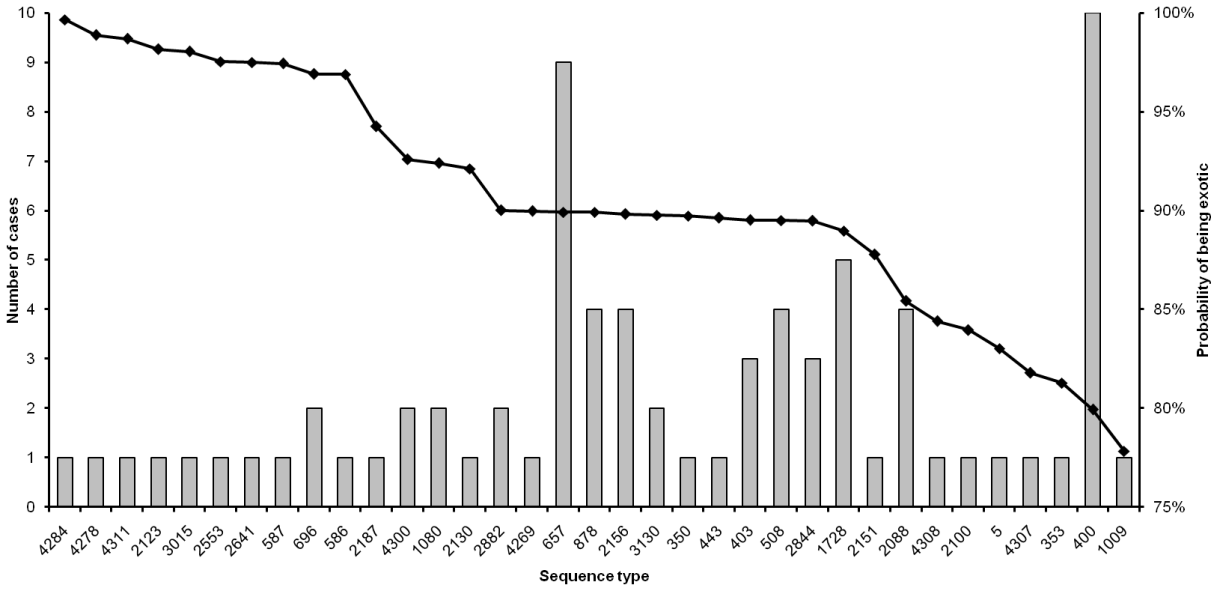

Figure 3. Number of cases (bar chart, left y-axis) and estimated probability (line chart, right y-axis) of the sequence types included in the risk factor analysis for domestically-acquired campylobacteriosis of probable exotic origin.

4. DISCUSSION

The risk for Dutch travelers to acquire campylobacteriosis depended on the travel destination. Consistent with previous studies [2,3], Asia, Africa, Latin America and the Caribbean, and Southern Europe were regions associated with an increased risk for campylobacteriosis compared to Western Europe, which comprises the neighboring countries of The Netherlands. Regional risk variations may be due to differences in local epidemiology and hygiene standards. However, a cautious interpretation of these risk figures is warranted, as physicians may be more likely to decide on laboratory investigation when a gastroenteritis patient has traveled to a high-risk destination [2].

Table 3. Sequence types found exclusively in travelers returning to The Netherlands but not in cases acquired in The Netherlands, nor in any of the sourced animal and environmental reservoirs.

Sequence type	Number of cases	Travel destination
4291	1	Austria
474	1	Belgium
4274	1	Bolivia, Peru
3878	1	Czech Republic
4252	1	Czech Republic
830	1	Egypt
931	1	Egypt
2229	1	Egypt
2968	1	Egypt
3606	1	Ethiopia
892	1	France
1044	1	Germany
446	1	India
451	1	India
1042	1	India
4288	1	India
530	2	Indonesia
161	1	Indonesia
2031	1	Indonesia
2109	1	Indonesia
2131	1	Indonesia
2393	1	Indonesia
2941	1	Indonesia
4270	1	Indonesia
4281	1	Indonesia
4287	1	Indonesia
4289	1	Indonesia
4296	1	Indonesia
4298	1	Italy
4305	1	Jordan
3630	1	Jordan, Iraq
1380	1	Kenya
466	1	Luxemburg, France
4293	1	Malaysia
1039	1	Mali
4309	1	Mali
2116	1	Morocco
3575	1	Morocco
4165	1	Morocco
4299	1	Morocco
986	1	Nepal, China
1233	1	Peru
4053	1	Peru
2895	1	Philippines
614	1	Poland
4277	1	Portugal
904	2	Portugal, Spain
4275	1	Singapore
148	1	Spain
1710	1	Spain
4294	1	Spain
4313	1	Spain
4408	1	Spain
1919	3	Thailand
768	2	Thailand
407	1	Thailand
1953	1	Thailand
2083	1	Thailand
2315	1	Thailand
4303	1	Thailand
3246	2	Turkey
303	1	Turkey
305	1	Turkey
2066	1	Turkey
2184	1	Turkey
2275	1	Turkey
3142	1	Turkey
919	1	Vietnam, Malaysia

STs of travelers and non-travelers were relatively similar and travelers showed more ST diversity than non-travelers, a possible reflection of the numerous countries where they acquired infection. Moreover, some STs were more likely than others to infect travelers visiting specific regions, and travelers infected with STs that were undetected domestically had traveled predominantly to distant destinations in Asia and Africa, suggesting that differences in STs are related, to some extent, to the geographical distance of the travel region compared to The Netherlands, with STs from nearby European countries being generally more similar than those from farther destinations [13].

The larger ST diversity in travelers combined with the association of some STs with specific destinations is also consistent with the presence of heterogeneously distributed clones that are endemic in the different regions but not so prevalent elsewhere in the world. Regionally endemic STs have been identified, for instance, in Australia [29], New Zealand [30] and Curaçao [31], and may emerge because of clonal expansion, niche adaptation, geographical isolation and host immune selection [32]. Although so far there has been no evidence of ST-specific immune responses, it is conceivable that the chance of being exposed to a ST with uncommon antigens is somewhat higher for STs that are rarely, rather than commonly, encountered. STs that are associated with strong regional clustering would therefore pose a higher risk to the travelers also because of limited, if absent, prior (repeated) exposure in addition to issues related to sanitation failure. The risk posed by uncommon STs is also suggested by their age distribution [16,33]. For instance, the three commonest STs among non-travelers were mainly found in the young relatively to the other STs, decreasing steadily with age (data not shown). Conversely, rare STs (<5 isolates) occurred independently of age. According to interpretation of similar findings [11], it is likely that antigenic properties associated with the common STs are frequently encountered throughout life; thus, the young would be more susceptible because they have encountered these less often. In contrast, rare STs, more probably related to uncommon antigens, would have seldom been encountered by all age groups.

Seven risk factors for travel-related campylobacteriosis were identified. Consistent with evidence that poultry is the main reservoir for campylobacters, most studies concerning risk factors for campylobacteriosis have

identified an association with eating chicken [6,7,16,34,35], suggesting that this risk factor is not exclusive of acquiring infection abroad. This also applies to consuming antacids and having chronic enteropathies [6,7,16].

Table 4. Multivariable odds ratios (OR) and corresponding 95% confidence intervals (95% CI) of risk factors for *Campylobacter jejuni/coli* infection in travellers returning to The Netherlands.

Risk factor (% of imputed missing values)	OR (95% CI)**
Days stayed (3)	1.0 (0.9-1.1)^{ns}
Region of destination*	
Western Europe	Reference
Northern Europe	0.8 (0.3-2.2)^{ns}
Eastern Europe	1.1 (0.3-2.2)^{ns}
Southern Europe	1.7 (1.0-3.3)
Northern Africa	10.6 (2.3-49.0)
Sub-Saharan Africa	25.4 (2.7-310.7)
Western Asia	10.6 (2.8-39.9)
South-Eastern Asia and China	27.8 (4.5-170.9)
Southern Asia	28.9 (2.4-265.1)
Oceania	0.8 (0.0-43040.2)^{ns}
Latin America and the Caribbean	20.8 (2.0-211.6)
Eating chicken (2)	2.0 (1.1-3.5)
Eating yoghurt (4)	0.4 (0.2-0.7)
Eating vegetable salad (3)	
travelling within Europe	1.7 (0.9-3.1)^{ns}
travelling outside Europe	6.7 (2.1-40.2)
Drinking bottled water (3)	
travelling within Europe, excluding Southern Europe	1.5 (0.6-3.6)^{ns}
travelling to Southern Europe and outside Europe	2.3 (1.6-5.0)
Contact with raw pork (5)	6.2 (1.3-29.3)
Recent use of proton-pump inhibitors	14.6 (3.0-82.0)
Having a chronic gastrointestinal disease (5)	2.9 (1.7-4.8)
Working in the medical/healthcare sector (1)	0.4 (0.1-0.9)

ns = not significant (p>0.05). *See Table 1 for details. **Adjusted for age, sex, degree of urbanization, season and level of education. Estimates are based on 328 cases and 244 controls.

Table 5. Multivariable odds ratios (OR) and corresponding 95% confidence intervals (95% CI) of risk factors for *Campylobacter jejuni/coli* infection acquired in The Netherlands caused by strains of most likely exotic origin.

Risk factor (% of imputed missing values)	OR (95% CI)*
Contact with people with gastroenteritis outside the household (3)	2.2 (1.9-4.6)
Recent use of proton-pump inhibitors	9.5 (4.4-20.6)
Eating yoghurt (2)	0.3 (0.2-0.6)
Not washing hands after toilet visit	
in April-December 2002	6.8 (0.7-68.1)^{ns}
in January-April 2003	20.8 (1.9-233.4)
Being a school going child	
in July-September 2002 and January-April 2003	1.4 (0.5-3.8)^{ns}
in April-June 2002	3.4 (1.0-11.4)
in October-December 2002	4.0 (1.4-11.3)
Swimming in a public swimming pool (1)	
in April-September 2002 and January-April 2003	1.0 (0.4-2.1)^{ns}
in October-December 2003	3.7 (1.3-11.0)

ns = not significant (p>0.05). *Adjusted for age, sex, degree of urbanisation, season and level of education. Estimates are based on 77 cases and 3119 controls

In The Netherlands, <1% of domestically-acquired *Campylobacter* infections have been attributed to pigs [16]. Moreover, eating pork has been associated with a reduced risk for *C. coli* [6] and chicken-borne *C. jejuni/coli* [16] infections. Accordingly, Dutch retail pork has rarely been found contaminated with campylobacters [36]. The association with raw pork we found therefore suggests that pigs are an important reservoir (and pork an important exposure) of campylobacteriosis outside The Netherlands.

In contrast to previous findings indicating that eating vegetable salad protects against domestically-acquired campylobacteriosis [6,16], we observed that this factor was associated with an increased risk for campylobacteriosis when traveling outside Europe. In Europe, extensive sampling of raw vegetables, including ready-to-eat salads, has generally found no, or very few, campylobacters [36], suggesting that contamination of such items during irrigation, harvesting and processing is unlikely and that salads may occasionally become cross-contaminated during food preparation [34]. Conversely, exceptionally high *Campylobacter* isolation rates (~68%) in raw vegetables were reported from countries such as Malaysia [37], indicating that major problems can arise by consuming vegetables if hygiene practices are absent or break down.

Drinking bottled water was associated with an increased risk for campylobacteriosis when traveling to high-risk destinations. In the UK, drinking bottled water has been identified as a risk factor for campylobacteriosis [34], particularly *C. coli* infection [38], and ciprofloxacin-resistant *Campylobacter* infection acquired abroad [5], suggesting that bottled water could, given the right circumstances, provide a vehicle for campylobacters [34]. In fact, bottled water, unlike tap water, is not usually treated and testing for *Campylobacter* is rarely undertaken [34,38]. Moreover, in the event of dual contamination of bottled water (campylobacters and organic matter), *C. jejuni* may survive for prolonged periods [39]. However, our association with bottled water was only significant when traveling to high-risk destinations, supporting the hypothesis that drinking bottled water acts as a proxy for local circumstances where there is a generally high risk for campylobacteriosis. Travelers are indeed usually advised to drink bottled water where there is any doubt about the local water quality. The use of bottled water may help in preventing infection but there may be circumstances where the risk is higher than that which can be prevented by drinking bottled water. Moreover, our questionnaire did not distinguish between sparkling and still bottled water and did not ask whether it was consumed with or without ice. Therefore, further investigation is needed to assess if the advice of drinking bottled water merits any refinement.

Consuming yoghurt and working in healthcare were protective. It is believed that probiotic bacteria in yoghurt may alter the intestinal microflora in a way that prevents infection [35], while people working in healthcare might be particularly aware of the health risks (and ways to avoid them) when traveling.

Risk factors for exotically-introduced domestic campylobacteriosis were suggestive of anthroponotic transmission, namely: contact with gastroenteritis cases outside the household (thus less likely to share the same exposure); not washing hands after toilet visit; being a school going child (usually having high frequencies of contacts); and attending public swimming pools (as recreational water has been proposed as a vehicle for *Campylobacter* transmission [16,35]). Except for the first risk factor, the others were unidentified in previous analyses where the same cases were not split according to their estimated exoticism [6,16]. Moreover, we found significant interactions with season, which is in accordance with the seasonal nature of traveling, as also shown by the finding that Pr_e varies seasonally. Periods most at-risk were mainly those around popular holiday periods in The Netherlands, notably the autumn break in October, Christmas/New Year in December–January, and Easter in April–May. Moreover, people most at-risk were school-going children for which additional peaks in domestic campylobacteriosis have already been noted shortly after the end of school breaks, suggesting that these additional peaks are due to exposure to less common strains from less common foods consumed during the festivities and to the mixing of people that have not been in contact for a long time following on from the previous holidays [40]. It was therefore hypothesized that travelers infected with strains possessing uncommon antigens might still be shedding them after returning home, most likely asymptomatically [14]. As there is unlikely to be a high prevalence of acquired protection to these exotic strains domestically, there is at least the potential for them to spread even through limited person-to-person transmission.

In conclusion, we investigated MLST profiles of *C. jejuni*/*coli* strains isolated from travelers, the risk factors potentially responsible for acquiring such strains upon traveling, and those potentially responsible for their secondary spread to domestic populations. As travelers have dynamic interactions with people, places, and microbes during their journeys, they can be victims, carriers, and eventually transmitters of such agents to new regions and populations. Our understanding of campylobacteriosis may therefore depend on increased insight into *Campylobacter* transboundary epidemiology, including regional risk differences, high-risk exposures and *Campylobacter* behavior in response to newly available susceptible populations and changing environments.

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Chapter 9

General discussion and conclusions

General discussion and conclusions

1. OUTLINE

In this thesis, the sensitivity towards pathogens causing human gastroenteritis of two newly implemented regional surveillance systems in Italy was assessed, the occurrence and distribution of human *Salmonella* infections in Italy were explored, and sources, risk factors and transmission pathways of human salmonellosis (in Italy) and human campylobacteriosis (in The Netherlands) were investigated by developing source attribution models and tools for the combined analysis of source attribution and case-control data. This entailed considerable data mining and modelling work using different methodological approaches to target specific research questions. Evidence provided in this thesis can nicely integrate with, and further expand on, current knowledge in the field of epidemiology of salmonellas and campylobacters at the human-animal-environmental interfaces. As filling gaps in knowledge is the first step towards a comprehensive understanding of zoonotic enteric pathogens, this thesis is also expected to support evidence-based decision making on prevention and mitigation strategies for *Salmonella* and *Campylobacter* in the transmission chain.

Each of the chapters of this thesis is "self-conclusive", that is, intended to stand alone as a detailed presentation of the topic (introduction), of the methods used and results obtained, as well as their discussion and conclusions drawn. The purpose of this general discussion is therefore to tie together the various studies presented in the body of this thesis and to make general comments and conclusions upon their meaning in relation to the four objectives of this thesis listed in the general introduction (Chapter 1). This also includes communicating the implications resulting from these papers and, when appropriate, making recommendations, forecasting future trends and the need for further research.

2. OBJECTIVE 1 – EPIDEMIOLOGY AND SURVEILLANCE OF ENTERIC PATHOGENS IN ITALY, WITH A FOCUS ON HUMAN SALMONELLOSIS

The first objective of this thesis was to provide an overview of the epidemiology of acute gastroenteritis in Italy, with a focus on human salmonellosis, particularly of *S. enterica* subsp. *enterica* serotypes, and to identify the most promising changes to be made to improve the sensitivity towards pathogens causing human gastroenteritis of Italy's current surveillance systems.

The epidemiology of human salmonellosis in Italy was described through the occurrence and distribution of non-typhoid *Salmonella* infections in the Italian general population using two main sources of data: 1) the official notifications of cases reported by physicians to the Italian National Infectious Diseases Notification System (SIMI) and to the Italian National Institute of Statistics (ISTAT); and 2) the reports of *Salmonella* serotyped isolates from humans notified by a network of diagnosing laboratories to Enter-net Italia, the current Italian laboratory-based surveillance system for enteric pathogens. The analysis of these different data gave comparable results, but at different resolutions. Indeed, the SIMI/ISTAT data are not differentiated into *Salmonella* serotypes, while the Enter-net Italia data provide microbiological information (at least the serotype) on *Salmonella* isolates of approximately 50% of human cases of salmonellosis notified to the SIMI/ISTAT. The parallel analysis of these two sets of data was useful in defining the general epidemiological situation of human salmonellosis in Italy and provided an opportunity for us to generate hypotheses about the underlying factors driving the occurrence and distribution of human *Salmonella* infections in Italy.

Most results were expected, particularly those regarding the age distribution (skewed towards the young) and the seasonal pattern (peaking in warmer months) of human *Salmonella* infections. It was, however, informative to discover that the

top six *Salmonella* serotypes isolated from humans (i.e., *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Derby, *S.* Typhimurium monophasic variant 4,[5],12,:i:- and *S.* Napoli) accounted for 59% of all *Salmonella* isolates from humans. Also the observed decreasing temporal trend in human salmonellosis and the increasing one of non-*Salmonella* diarrhoeal infections are in line with the increasingly prominent role of pathogens other than *Salmonella*, such as campylobacters, which are the most frequent cause of human acute gastroenteritis in the EU [1]. Besides evidence that human salmonellosis as a whole has been decreasing significantly in Italy since the 1990s, passing from 47 to 7 cases per 100,000 population from 1992 to 2009, we also found that, since 2000, this decrease has mainly been driven by specific serotypes, such as *S.* Enteritidis and *S.* Infantis, whereas other serotypes have emerged (*S.* Typhimurium monophasic variant 4,[5],12:i:-, *S.* Derby and *S.* Napoli) or have remained fairly stable (*S.* Typhimurium) over time, suggesting that the applied control measures are not equally efficient against the different serotypes and that the sources of infection other than those of *S.* Enteritidis (laying hens and eggs) are probably becoming increasingly important. Indeed, the importance of the different sources of human salmonellosis may change over time [2], and failure to identify the most important reservoirs in space and time may result in relatively ineffective control measures and waste of resources. We also found that, since 2000 in Italy, human *S.* Enteritidis infections fell consistently below those caused by *S.* Typhimurium, which is the most reported serotype in Italy in contrast to the international situation where *S.* Enteritidis still ranks at the top despite its significant decrease. This finding further provoked our strong interest in identifying the main reservoirs of human salmonellosis in Italy, as well as quantifying their importance in terms of human infections attributed to different animal and food sources, providing risk managers with evidence to focus *Salmonella*-reducing control efforts along the transmission chain.

In sporadic cases of acute gastroenteritis, testing for *Salmonella* spp. and subsequent serotyping serve as the predominately used surveillance tool for reaching an aetiological diagnosis, monitoring trends over time, and attributing human infections to the different food and animal sources. *Salmonella* serotyping is therefore very useful for informing and addressing public health actions, providing also a basis for epidemiological research, especially regarding the emerging serotypes (which may reveal the presence of previously unrecognized sources of infection) and the efficacy of intervention strategies. However, it is generally recognized that underreporting to surveillance systems is massive and rather unbalanced over the various subgroups of the population. Because of this, there is still incomplete knowledge about the real incidence and distribution of human *Salmonella* infections in many countries, including Italy.

Surveillance of zoonotic enteric pathogens is usually conducted to: 1) facilitate the control of the disease through prompt public health actions; 2) measure the magnitude and trends of the disease; 3) improve and update our knowledge of the determinants, sources, reservoirs, risk factors, transmission routes, morbidity and mortality of the disease; 4) guide intervention strategies and their evaluation; and 5) assist policy makers in setting priorities. To date, the SIMI (and Enter-net Italia as well) is able to address only a few of these points. By evaluating the impact of the two newly implemented surveillance systems in Lombardy and Piedmont regions on their overall notification rate of acute gastroenteritis cases and food-borne disease outbreaks, we also aimed at identifying the most promising directions to improve reporting at the national level. We found that, compared with the national mean, data from Lombardy and, to a lesser extent, Piedmont showed a significant increase in notification rates of human cases of both non-typhoid salmonellosis and non-*Salmonella* infectious diarrhoea, but for food-borne disease outbreaks, the increase was not statistically significant in none of the two regions. However, a further study [3] on this subject using a more sophisticated statistical analysis has found a significant increase in food-borne disease outbreaks in Piedmont region. It was therefore concluded that these two regional systems have improved their sensitivity regarding notification of acute gastroenteritis cases and food-borne disease outbreaks, thereby providing a more complete picture of the epidemiology of these diseases in Italy.

The "positive" impact of the implementation of the regional surveillance systems on acute gastroenteritis notification rates was, however, considerably more evident in Lombardy than in Piedmont. It follows that,

at least in principle, the system of Lombardy provides a more promising (regional) example to be emulated at the national level in order to improve reporting of acute gastroenteritis cases and consequently obtain a better estimate of their occurrence in the Italian population. Nevertheless, the system of Piedmont, which is dedicated to food-borne pathogens and specifically to early warning of food-borne disease outbreaks, allows for a broader collection of information that is not easy to obtain in other ways, and this is indeed particularly relevant for timely outbreak detection, investigation, prevention and response.

In order to improve the surveillance of acute gastroenteritis at the national level by looking at the experience gained at the regional level, such as that of Lombardy and Piedmont, it is also necessary to consider the feasibility, cost-benefit and long-term sustainability (i.e. the resources needed to sustain the system in the long-term) of the changes to be made to the national system in addition to the desired/expected outcome in terms of underreporting reduction. Nonetheless, by merely looking at the desired/expected outcome, there is some suggestive evidence indicating that the sensitivity towards pathogens causing gastroenteritis of the Italian surveillance system might benefit from careful consideration of extending the Lombardy, and to a somewhat lesser extent, Piedmont systems to the other regions. Efforts should be focused on the integration and harmonization of different surveillance activities and sources of information, as well as evaluation of such activities, to obtain the best achievable impact on the burden of acute gastroenteritis in the population. There is also an urgent need for surveillance of enteric pathogens in Italy to work, at the same time, on isolating, identifying, and reporting of detailed typing findings for both diagnostic and public health purposes.

3. OBJECTIVE 2 – ATTRIBUTION OF HUMAN SALMONELLOSIS TO ANIMAL AND FOOD SOURCES IN ITALY

The second objective of this thesis was to develop source attribution models based on the microbial subtyping approach using Italian *Salmonella* data in order to estimate the relative contributions of different animal and food sources to human *Salmonella* infections in Italy. Moreover, we were interested in investigating the changes in attribution estimates over different models, time periods and attribution points along the farm-to-fork continuum.

The modified Hald model [4] was adapted for source attribution of human salmonellosis in Italy. An original model that we called "modified Dutch model" was also developed and applied to the same data (and for the same purposes) as those of the modified Hald model. Both these models allowed for the estimation of the relative contributions of different animal and food sources, as well as outbreaks and foreign travel, to laboratory-confirmed human *Salmonella* infections in Italy from 2002 to 2010. The methodological approach we used is, however, a flexible one, and could therefore be extended to other pathogens and countries in the near future.

With some differences in consistency and precision of attribution estimates over time periods and sampling points, both our model adaptations identified pigs as the main source of human salmonellosis in Italy, accounting for approximately half of human cases, followed by *Gallus gallus*, whereas the contributions of turkeys and ruminants were only minor. This ranking provided significant insights about the sources that are being increasingly important in Italy, providing a basis for delineating future risk management strategies. Indeed, while the applied control measures seem to have worked well in poultry, it became apparent that there is an urgent need to focus attention on pigs. This is also substantiated by recent findings indicating that pigs are the most important source of human salmonellosis in other European countries, such as Belgium, Cyprus, Finland, France, Ireland, Poland and Sweden in addition to Italy, with also very similar proportions of cases attributed to poultry and to pigs in the Netherlands [5].

The increasingly prominent role of pigs, and the decreasingly one of *Gallus gallus*, as sources of human salmonellosis in Italy nicely integrate with evidence provided in Chapters 3. Indeed, in Italy and in most other industrialized countries, there has been a drastic decrease in the number of human *S.* Enteritidis infections (for which *Gallus gallus*, and particularly layers, are the major reservoir [5,6]), whereas a sustained predominance of infections caused by *S.* Typhimurium and its monophasic variant 4,[5],12,:i:-, and an increase of those caused by *S.* Derby, have been observed. Accordingly, *S.* Typhimurium,

its monophasic variant 4,[5],12,:i:- and *S.* Derby entail pigs as the main reservoir [5,6]).

Data used in our model adaptations were probably suboptimal for source attribution in some respects. This is a reflection of 1) deficiency in data availability, such as the lack of reliable *Salmonella* phage typing data for the commonest serotypes in humans and sources; 2) comprehensive travel and outbreak information, as well as detailed prevalence data in each of the sources; 3) the low number and resolution of sources surveyed, particularly *Gallus gallus*, which is not subdivided in at least broilers and layers; 4) no information on imported foods, nor on vegetables, which, to our knowledge, have never been considered in this kind of source attribution studies notwithstanding their increasing importance as a source of human salmonellosis [7–9]. Not to mention some special low-moisture foodstuffs, such as peanut butter, infant formula, chocolate, cereal products and dried milk, through which salmonellas can be delivered as well [10].

The modified Dutch model represents a practical improvement of the original Dutch model [11], which, in spite of its simplicity and easiness of application, has the major disadvantage of not accounting for differences in the ability of the different subtypes and sources to cause disease in humans. This means that, within each microbial subtype, the original Dutch model assumes that the impact of every source is equal and proportional to the occurrence of that subtype in that source. Similar to the approach on which the (modified) Hald model relies, two major modifications were applied to our modified Dutch model. First, the prevalence was modelled using the methodology of Mullner *et al*. [4], which allowed us to take into account the overall probability of finding *Salmonella* in a given source in addition to the relative frequency of the different serotypes within each source. By doing so, the model can now make use of the best possible estimate of the prevalence. Second, as reliable food consumption data were available and environmental, anthroponotic or unknown sources were not included in the model, food consumption weights were incorporated to take into account the human exposure to the different sources. By incorporating food consumption data, the model is better informed and can more closely reflect the chance of a given source to act as a vehicle for *Salmonella*. It follows that, similar to the (modified) Hald model, our modified Dutch model no longer assumes an equal impact of the different subtypes and source on the human disease burden.

In general, the conclusions reached by the two models applied at farm and food levels fundamentally agree with one another, and therefore provided little information to support risk managers in identifying the most promising targets along the farm-to-fork continuum on which control efforts should be focused. Discrepancies in the estimates between the two models may be explained by the different computational methods they use, as evidenced elsewhere [12–4]. Moreover, it is still rather unclear if the considerable increase in the contribution of *Gallus gallus* to human *Salmonella* infection from farm to food estimated by our modified Hald model is due to an important role played by leaks in hygiene practices along the food chain for chicken meat and eggs in modifying the within-source serotype distribution in such a way that the "risk" posed by *Gallus gallus* increases considerably at food level relative to that at farm, or is instead due to a particular sensitivity of this kind of model to biologically meaningless changes in within-source serotype distribution. As we could not determine here to what extent the changes (from farm to food) in the within-source serotype distribution we found were biologically meaningless, further investigations are needed.

The serotype (q_i) and source (a_j) dependent factors of the (modified) Hald model merit special considerations. These factors should describe complex systems that are not still fully understood. It is assumed that q_i accounts for differences in survivability (along the food chain) and pathogenicity (in humans) of the various *Salmonella* subtypes. Similarly, a_j is assumed to account for variability in surveillance systems and for specific characteristics of the sources that allow them to act as vehicles for salmonellas. However, the method relies on a sort of "black box" model [2], and values taken by q_i and a_j are only assumed to be a summary reflection of unknown biological properties.

In conclusion, attributing human *Salmonella* infections to animal and food sources in Italy is a valuable tool to quantify and rank their relative importance for human disease, and thus expected to support risk management decisions, assist prioritization of interventions, and help measuring the effect of control programmes in the near future. As

higher quality data from a fully integrated (human and animal) intensive surveillance system are needed to improve our attribution estimates, new developments in this field should work in parallel on empowering surveillance efforts and facilitating the application of source attribution analyses in the presence of imperfect surveillance data.

4. OBJECTIVE 3 – COMBINING SOURCE ATTRIBUTION AND CASE-CONTROL DATA ON HUMAN CAMPYLOBACTERIOSIS, WITH CONSIDERATIONS ON SAMPLING ISSUES

The third objective of this thesis was to develop a combined analysis of source attribution and case-control data on human campylobacteriosis while accounting for sampling issues arising from source attribution in space and time.

MLST is increasingly becoming the typing method of choice in source attribution studies of human campylobacteriosis, e.g. [4,14–16]. However, the implementation of intensive sampling schemes to obtain representative *Campylobacter* MLST datasets from multiple sources is rather expensive. Therefore, for the purposes of *Campylobacter* source attribution, investigators might be forced to use non-recent or non-local MLST data, which can potentially introduce bias in the attribution estimates [17]. Moreover, the use of small-sized datasets may result in uncertain estimates. Although there is evidence that *Campylobacter* multilocus genotypes are more strongly associated with specific hosts than with geographical location [18], there are still large geographical differences in the distribution of host-associated genotypes, and these can change over time as well [19]. This highlights the need to consider concurrent sampling of different reservoirs in time and space in parallel to that of humans. Other issues that may need further considerations are: 1) which reservoirs to sample; 2) whether and how to sample the environment (e.g. water sources) as a proxy for unknown reservoirs; and 3) which genotyping tools to use in alternative to MLST. Within-country variation may also be considered when deciding whether to adopt a whole-country approach or to use sentinel sites [4]. At present, there are few indications about the impact of (spatial and temporal) genotype variation and sample size on source attribution estimates. This provoked our interest in investigating the consequences of using non-local or non-recent MLST data when attributing human *Campylobacter* infections to putative sources (i.e., chicken, cattle, sheep, pig and the environment). We have therefore performed a series of analyses aimed at determining how the Asymmetric Island (AI) model for source attribution performs in absence of local or recent data (by supplementing or substituting source data with data from other countries and/or time periods) and when only few data are available (power analysis). The importance of geographical distance in *Campylobacter* multilocus genotype dissimilarity was provided by comparing human MLST datasets from several countries to the Dutch ones. Indeed, data from nearby European countries were generally more similar than data from more distant countries with respect to The Netherlands. This is also in agreement with that we found in the study of travel-related *Campylobacter* infections presented in Chapter 8. Evidence was also found for a shift in ST frequencies over time.

As MLST data become increasingly dissimilar as the geographical distance and the time period between different datasets are collected increases, the AI model can underestimate the importance of a source whose data are not collected contemporaneously with the human cases to be attributed. Indeed, although chicken was identified as the most important source of human campylobacteriosis in The Netherlands, accounting for 61-74% of cases, this high proportion of chicken-attributed cases (and the smaller ones attributed to non-chicken sources) depended on the origin of the source data included in the AI model. Generally speaking, the farther in space and time one takes the source data, the more their MLST profiles will differ, and the smaller will be the estimated proportions of human cases attributable to those sources. Nevertheless, there is also evidence that the extent of the bias introduced by temporal mismatching between human and source data is much smaller than that introduced by geographical mismatching. We therefore proposed a coarse rule stating that this bias increases with the geographical distance between the countries (and to a lesser extend with the temporal distance between the time periods) from which source data are used. However, our results also suggest that geographical distance may act together with

factors related to travel and trade between countries, as also evidenced in Chapter 8. These findings are expected to be practical in guiding sampling schemes for *Campylobacter* MLST data collection in future source attribution studies where the potential for geographical and/or temporal bias cannot be ignored. In general, however, the extent to which such bias is a matter of concern depends on how detailed in time and space is the research question to be addressed.

We also proposed a very practical method, which is a sort of "poor man's solution", to select supplementary non-local or non-recent MLST source data with the aim of minimizing the potential geographical and temporal biases. This method is based on the assumption that if the human MLST data between different countries and time periods resemble one another (as revealed by PSI or principal component analysis - PCA), then also will their respective source MLST data, which may therefore be borrowed interchangeably from the other datasets in question. Finally, our results also suggest that it is recommendable to have over 100 isolates per source to perform source attribution using the AI model in order to have satisfactory statistical power. More detailed research questions might, however, ask for more precision, i.e. a larger strain set.

Blending the properties of source attribution models and case-control studies was thought to be useful for risk management and prioritization of control strategies. This is because case-control studies alone are insufficient for attributing human infections to the different reservoirs, as they can only trace back the source of infection to the points of exposure (e.g. food items consumed), which may not point to the original (amplifying) reservoirs because of cross-contamination. Human *Campylobacter* infections can, however, be attributed to specific reservoirs using source attribution models based on MLST data, such as the AI model. Combining case-control data with the results of the attribution analysis would therefore allow us to explore risk factors at the point of exposure for human campylobacteriosis caused by strains originating from the different reservoirs, thereby tracing the transmission route from the exposure up to the reservoir, and vice versa. This may greatly improve our knowledge about the identification and characterization of potential reservoirs, risk factors and transmission pathways for human campylobacteriosis, as well to generate hypotheses and corroborate, to some extent, if MLST-based source attribution makes sense epidemiologically.

Our combined analysis is based on the application of the AI model addressing the aforementioned sampling issues to estimate a probabilistic reservoir assignment (posterior probability) for each *Campylobacter* ST isolated from human cases, and using these attributed cases as outcome in the case-control study. This is an extension of earlier case-case comparisons of poultry- and ruminant-associated cases of human campylobacteriosis [20,21] to include information on non-diseased controls, which is likely to identify more subtle associations and in turn improve source attribution modelling. We therefore investigated risk factors for human campylobacteriosis caused by STs with the highest possible probability to originate from chicken, ruminants (cattle and sheep) and the environment, considered as a proxy for other unidentified reservoirs. Once again, results revealed that most human cases were attributed to chicken (66%), followed by cattle (21%), which were therefore identified as the main reservoirs of human campylobacteriosis in The Netherlands. Moreover, our results provided suggestive evidence that chicken is the major reservoir for campylobacteriosis in young children living in urban areas compared with their rural counterparts, for which cattle seems to be more important.

While the attribution analysis quantified the relative contributions of the considered reservoirs to human infections, the risk factor analysis identified the excess risk exposures for infections that were highly associated with these reservoirs, as well as their quantification in terms of population attributable risk (PAR). For instance, only up to 42% of the highly chicken-associated infections could be ascribed to consumption of chicken, suggesting that a considerable part of infections originating from chicken is acquired by pathways other than food, such as the environment or even by cross-contamination to commodities, utensils, and foods other than chicken. As expected, consuming chicken was identified as a risk factor for human campylobacteriosis caused by chicken-associated STs, whereas consuming beef and pork were protective. Risk factors for human campylobacteriosis caused by ruminant-associated STs were contact with animals, barbecuing in non-urban areas, consumption of tripe, and never/seldom consumption of

chicken, while consuming game and swimming in a domestic swimming pool during springtime were risk factors for human campylobacteriosis caused by environment-associated STs. Infections with chicken- and ruminant-associated STs were, however, only partially explained by food-borne transmission; direct contact and environmental pathways were also important.

Evidence provided by these results indicates that human campylobacteriosis in The Netherlands (but probably also in other countries) could greatly be reduced by focusing interventions on chicken and cattle, especially in urban and rural areas, respectively. However, pathways alternative to the food-borne one, such as direct contact and environmental contamination, do play a role as well, particularly in ruminant-associated infections. We also demonstrated that risk factors for *Campylobacter* infection depend upon the attributed reservoirs and that the exposure may plausibly direct to the original reservoirs when considering those STs that are indeed highly associated with the reservoirs in question. This provided a novel framework to support and generate hypotheses about *Campylobacter* epidemiology at the human-animal-environmental interfaces and, in a broader perspective, to corroborate that the general concept of MLST-based source attribution modelling for campylobacteriosis is epidemiologically sensible.

A number of case-control studies have explored risk factors for *Campylobacter* infection at the point of exposure while other studies have used MLST data to attribute *Campylobacter* infections to animal or environmental reservoirs. Our approach is innovative as it attempts to bridge this gap by exploring risk factors at the point of exposure for human campylobacteriosis of different origins, using a combined case-control and source attribution analysis. Obviously, this approach is not free of major caveats. For instance, some risk factors may be significantly associated with infections attributed to a given reservoir just because these infections have a residual contribution (i.e. attribution) from reservoirs other than those to which they were attributed. This residual contribution, although minimized through the selection of the most host-associated STs, creates "noise" which could have masked or diluted some associations, or led to some additional associations, in the risk factor analysis. Nevertheless, all the risk factors we found were associated in an epidemiologically plausible way according to the reservoirs in question, which is an indication that the risk of spuriousness was handled fairly well.

Two other interesting findings were: 1) dog ownership as a risk factor for environment-associated strains; and 2) risk for *Campylobacter* person-to-person transmission particularly pronounced for environment-associated STs. It is clear that dogs are often carriers of campylobacters and that dog owners may be particularly exposed to *Campylobacter* strains of environmental origin while walking their dogs, and dogs may also act as a vehicle for *Campylobacter* strains of environmental origin. Moreover, considering that our environmental strains were sourced from water and sand among others, and that person-to-person transmission seems particularly important in children [22], we speculated that sand (particularly the one in playground sand-boxes) and recreational water can act as a vehicle for transmission among humans as well. These findings further provoked our interest in elucidating the role of pets in *Campylobacter* zoonotic transmission, as well as the role of potential anthroponotic sources, as presented in Chapters 7 and 8.

Enhanced models and genotyping tools, as well as the integration of different approaches, e.g. epidemiological and genealogical modelling, in a single framework have the potential for improving the range of techniques available for source attribution in the near future. Molecular subtyping tools may also be improved with the addition of whole genome sequence data from high through-put sequencing platforms, particularly when combined with improved bioinformatics and Web-based database tools that input short read sequence data. These have already led to the development of extended and generic MLST schemes [23,24]. Exploring the genome evolution of epidemiologically relevant strains may also improve the discrimination of unclear reservoirs of human campylobacteriosis, such as cattle and sheep, and result in more precise attribution estimates. Moreover, the identification of genetic markers for resistance to bacterial stress could also help in determining the sources and transmission routes of *Campylobacter* strains isolated from humans, further refining attribution studies and possibly stimulating novel epidemiological insights towards reservoir-specific risk factors

and transmission pathways for human campylobacteriosis.

5. OBJECTIVE 4 – EXTENDING THE APPLICATION OF COMBINED SOURCE ATTRIBUTION AND CASE-CONTROL MODELLING

The fifth objective of this thesis was to extend the novel application of the combined source attribution and case-control analysis presented in Chapter 6 to include also factors that are not commonly considered when examining likely sources of human campylobacteriosis, such as zoonotic transmission through and/or from pets (dogs and cats) and potential (secondary) anthroponotic transmission involving returning travellers. Indeed, standard frameworks for (food- and environment-borne) *Campylobacter* source attribution studies rarely consider the potential impact of these atypical transmission routes with anything more elaborated than a quick note in the discussions of published journal articles in which such impact is assumed to be minimal based on empirical evidence. However, person-to-person spread could be included by considering humans as a spill-over host [25] infected from an animal reservoir. This is similar to the role played by imported food, which is still part of the food pathway, but with the reservoir located abroad. Moreover, some original reservoirs may be repeatedly infected by other reservoirs, effectively acting as an intermediate host instead of a real maintenance host. For example, pets may be repeatedly infected from food animals, but not be the primary amplifying hosts. This may also be the case of human infections associated with pet ownership, which may be the result of direct contact with pet food contaminated by food animals, rather than the pet itself. It is clear that such complex feedback loops representing transmission between reservoirs are often omitted in regular source attribution studies to keep this already intricate framework relatively simple. However, it should be recognized that these transmission loops may be important when considering possible interventions [6].

To elucidate the role of pets, a poorly characterized reservoir of human campylobacteriosis, we first investigated MLST profiles of *C. jejuni* and *C. coli* strains isolated from pets and their owners in a one-to-one relationship and then extended our combined source attribution and case-control analysis to explore risk factors at the point of exposure for human campylobacteriosis of probable pet origin. Results revealed a high degree of overlap between *Campylobacter* STs of human and pets, and dog owners, especially puppy owners, had an increased risk for infection with STs associated with pets compared to controls and non-dog owners. Furthermore, the expected occurrence of identical STs in humans and their pets in a one-to-one relationship was significantly lower than that observed. Taken together our results suggest that dog ownership increases significantly the risk of acquiring *Campylobacter* infection caused by STs originating from pet and that co-isolation of identical strains in humans and their pets occurs significantly more often than expected by chance. We therefore envisaged four possible scenarios: 1) humans and pets become infected from the same source; 2) humans and pets become infected from different sources that incidentally carry the same strain; 3) humans become infected from dogs; 4) dogs become infected from humans. As the sampling design was non-directional in the transmission of infection, our results support evidence for genetic association of *Campylobacter* strains between humans and their pets but do not prove that transmission of such strains occurs from pets to humans, or vice versa. Although directionality of transmission could not be inferred, the combined analysis and the co-isolation of identical STs in pets and their owners proved that dog (particularly puppy) ownership is a risk factor for human *Campylobacter* infection caused by STs of probable pet origin and that co-isolation of identical strains in humans and their pets occurs more frequently than expected. It is still unclear to what extent this increased risk in dog owners is an indication of other unmeasured factors, such as owner's personality traits, lifestyle, income, disability or other health problems, which can plausibly influence the chance of becoming infected and the decision and manner of owning a pet. It is therefore recommendable to put the zoonotic risk posed by pets into context, depending on factors such as level of *Campylobacter* carriage and intensity and type of contact between pets and humans.

We also noted that attributing human infections to pets using the AI model may be misleading when the goal is to identify the original reservoirs, as pets may artificially

account for an abnormal amount of cases just because they are, like humans, predominantly endpoint hosts for campylobacters (as humans and pets share many sources of infection). Conversely, the contribution of the other reservoirs included in the model will be underestimated and probably biased towards those reservoirs from which pets acquire infection.

To elucidate the role of travellers, a possible "anthroponotic source" of campylobacters, we investigated: 1) the MLST profiles of *C. jejuni* and *C. coli* strains isolated from travellers returning to The Netherlands in comparison with those isolated from domestically-acquired cases; 2) the risk factors for travel-related campylobacteriosis by comparing the exposures of the returned travellers with those of travellers in the enrolled control population; and 3) the risk factors for domestically acquired campylobacteriosis caused by strains of probable exotic origin (putatively carried by the returned travellers) by applying our combined source attribution and case-control analysis.

Travellers are known to be particularly prone to experiencing symptomatic *Campylobacter* infections when travelling abroad as partial immunity to (severe) disease is acquired over time with repeated exposure to local *Campylobacter* strains. Indeed, there is some evidence suggesting that the disproportionately higher risk of campylobacteriosis in international travellers is not limited to higher levels of exposure in developing countries, but also to the possible presence of "new" (for the travellers) *Campylobacter* strains that are endemic in the different travel regions (strong regional clustering) and to which travellers have hardly been exposed before [27]. It follows that, probabilistically, these "new" strains are more likely to be associated with regionally untested antigens than widespread strains, and acquired protection may be ineffective when exposed to uncommon strains, as evidenced by a recent Canadian study [28]. We therefore hypothesized that when returning to the original countries, the infected, but not necessarily symptomatic [27], travellers, may introduce into the domestic population several "exotic" strains with a higher probability of possessing antigens that are underrepresented in the local reservoirs, i.e. food-producing animals, pets and wildlife. These exotically introduced strains would therefore have at least the potential to spill-over into the domestic population and at first spread anthroponotically.

Results revealed, again and convincingly, that travelling to Asia, Africa, Latin America and the Caribbean, and Southern Europe is associated with an increased risk for campylobacteriosis compared to travelling to Western Europe, which comprises the neighbouring countries of The Netherlands. STs of travellers showed more ST diversity than non-travellers (domestically acquired infections), and some STs had a significant regional clustering. Moreover, travellers infected with STs that were undetected domestically had travelled predominantly to distant destinations, suggesting that differences in STs are related, to some extent, to the geographical distance of the travel region compared to The Netherlands, as also evidenced in the analysis presented in Chapter 5. The larger ST diversity in travellers combined with the association of some STs with specific destinations is consistent with the presence of heterogeneously distributed clones that are endemic in the different regions but not so prevalent elsewhere in the world. Although so far there has been no evidence of ST-specific immune responses, it is conceivable that the chance of being exposed to a ST with uncommon antigens is somewhat higher for STs that are rarely, rather than commonly, encountered. STs that are associated with strong regional clustering would therefore pose a higher risk to the travellers also because of limited, if absent, prior (repeated) exposure in addition to issues related to sanitation failure. This hypothesis nicely fits with existing knowledge about acquired immunity to campylobacters with repeated exposure over time, as also suggested by our age distribution of STs.

From a preventive point of view, these results highlight the considerable potential value of *Campylobacter* vaccines for humans. This potential would relate to the prevention of acute infection and, most importantly, sequelae, which would lead to a greater reduction in the burden of disease. While vaccines are unlikely to be used in a prophylactic role for the general public, they would have a value for high-risk groups, such as travellers or military troops. However, considerable research is required before this potential can be realized. Indeed, in the past years, considerable research efforts have been made in both the public and private sectors to

develop new diarrhoeal disease interventions, including vaccines against rotavirus, cholera, typhoid, enterotoxigenic *E. coli* and *Shigella*. However, currently there are no approved vaccines or drugs that prevent *Campylobacter*-associated traveller's diarrhoea. Obviously, antibiotics can treat illness, but cannot prevent it effectively, resulting in decreased productivity of travellers. Moreover, antibiotic treatment may have the unintended consequence of contributing to increased antimicrobial resistance.

Besides universal risk factors for campylobacteriosis, such as eating chicken, using antacids, and having chronic gastrointestinal diseases, we also identified eating vegetable salad outside Europe, drinking bottled water in high-risk destinations (a proxy for local circumstances where there is a risk for campylobacteriosis higher than that which can be prevented by drinking bottled water), and contacting raw pork as specific risk factors for travel-related campylobacteriosis.

Risk factors for domestically-acquired campylobacteriosis caused by exotic STs involved predominantly person-to-person contacts around popular holiday periods. It was therefore hypothesized that travellers infected with strains possessing uncommon antigens might still be shedding them after returning to The Netherlands, most likely asymptomatically. As there is unlikely to be a high prevalence of acquired protection to these strains domestically, there is at least the potential for these exotically introduced strains to spread even through limited person-to-person transmission.

Investigating MLST profiles of *C. jejuni*/*coli* strains isolated from travellers, the risk factors potentially responsible for the acquisition of such strains upon travelling, and those potentially responsible for their secondary spread to domestic populations, was useful in expanding our understanding of regional risk differences, high-risk exposures in varying epidemiological dimensions, and *Campylobacter* behaviour and survival strategies in response to newly available susceptible populations and changing environments. We concluded that risk factors for travel-related campylobacteriosis differ from those for domestically acquired infections. There is also suggestive evidence that returning travellers may play an important role in *Campylobacter* epidemiology by carrying several exotic strains that might subsequently spread to domestic populations.

In extending the application of source attribution modelling to previously scarcely explored sources, we have discovered a mine of novel insights to expand our knowledge and generate hypotheses about *Campylobacter* (transboundary) epidemiology at the human-animal-environmental interfaces. Ultimately, by enhancing our ability to characterize the risk for human campylobacteriosis, public health initiatives can be better informed.

Future challenges of extended source attribution analyses will be the consideration of sources of particular subsets of human cases, such as those with infections resistant to antimicrobials and those associated with particular sequelae, such as Guillain-Barré syndrome. Moreover, methods based on microbial subtyping could be used to model the relative contribution of reservoirs contaminating particular pathways, such as surface water supplies to water treatment plants. Similarly, molecular epidemiological techniques using similar modelling approaches could be used to understand transmission cycles in primary production. This will require the conduction of newly tailored epidemiological studies and modelling approaches for the years to come.

6. CONCLUSIVE CONSIDERATIONS

Salmonella and *Campylobacter* are the most common zoonotic bacterial causes of human gastroenteritis in the world, causing considerable morbidity, mortality and economic impact. It is expected that these pathogens will continue to be of paramount importance in the future, as the global population moves toward animal products as a primary source of proteins. On a global scale, the distribution of such pathogens is also expected to be influenced by increased international trade and travel.

The epidemiology of salmonellas and campylobacters is complex; thus, a multi-tiered approach to control is needed, taking into account the different reservoirs, pathways, exposures and risk factors involved. Most recent epidemiological research conducted on salmonellas and campylobacters has been focused on *S.* Enteritidis and *S.* Typhimurium, and on *C. jejuni* and *C. coli*, whereas relatively little is known about the epidemiology of the other *Salmonella* serotypes and *Campylobacter* species with zoonotic potential. In public health terms, there is already a sufficient

evidence base to deal with the burden of *S.* Enteritidis, *S*. Typhimurium, *C. jejuni* and *C. coli* infections, whereas the importance of other *Salmonella* serotypes and *Campylobacter* species, although unclear, is unlikely to eclipse that of these pathogens.

Good surveillance is the starting point for studies of burden of disease and source attribution aimed at providing the evidence base that drives the need for control measures across all outcomes of human salmonellosis and campylobacteriosis. Recent developments in source attribution modelling and the ever-increasing number of countries conducting integrated laboratory-based surveillance is expected to result in significant advances in epidemiological research on various food-borne pathogens as well as in improved (evidence-based) control programmes.

Food-borne disease control needs to be adapted to local possibilities, practicalities and preferences. Some basic principles, however, are generally applicable and recommendable (e.g. biosecurity). While, historically, the primary target for *Salmonella*- and *Campylobacter*-reducing control programmes has been the poultry sector and, to a lesser extent, pigs and ruminants, some (emerging) transmission vehicles, such as raw milk, fresh produce and drinking water are in urgent need of attention. Although poultry is the historical source of *Salmonella* and *Campylobacter* infection in many countries, there is evidence that controlling such pathogens in poultry will not completely eliminate the problem. Options are available to target control pathways other than poultry.

It is difficult to trace sources of *Campylobacter* and, to a lesser extent, *Salmonella* infections because of their apparently sporadic nature and the important role of cross-contamination. Yet, many countries working to prevent food-borne salmonellosis and campylobacteriosis have made considerable progress on numerous fronts during the past years. Technological and scientific advances moving towards rapid, high-throughput, comprehensive analytical methods offer new approaches, such as whole genome sequencing. It is highly likely that most classical phenotyping techniques will be soon replaced by inexpensive and less labour-demanding genome-based tools. Source attribution studies are therefore expected to adopt a more holistic attitude, integrating different approaches, considering multiple sources and pathways of exposure but also relying on (genotypic) data with enhanced discriminatory power.

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General summary and acknowledgements

1. GENERAL ABSTRACT

Salmonella and *Campylobacter* are common causes of human gastroenteritis. Their epidemiology is complex and a multi-tiered approach to control is needed, taking into account the different reservoirs, pathways and risk factors. In this thesis, trends in human gastroenteritis and food-borne outbreak notifications in Italy were explored. Moreover, the improved sensitivity of two recently-implemented regional surveillance systems in Lombardy and Piedmont was evidenced, providing a basis for improving notification at the national level. Trends in human *Salmonella* serovars were explored: serovars Enteritidis and Infantis decreased, Typhimurium remained stable and 4,[5],12:i:-, Derby and Napoli increased, suggesting that sources of infection have changed over time. Attribution analysis identified pigs as the main source of human salmonellosis in Italy, accounting for 43–60% of infections, followed by *Gallus gallus* (18–34%). Attributions to pigs and *Gallus gallus* showed increasing and decreasing trends, respectively. Potential bias and sampling issues related to the use of non-local or non-recent multilocus sequence typing (MLST) data in *Campylobacter jejuni*/*coli* source attribution using the Asymmetric Island (AI) model were explored. As MLST data become increasingly dissimilar with increasing geographical and temporal distance, attributions to sources not sampled close to human cases can be underestimated. A combined case-control and source attribution analysis was developed to investigate risk factors for human *Campylobacter jejuni*/*coli* infections of chicken, ruminant, environmental, pet and exotic origin. Most infections (~87%) were attributed to chicken and cattle. Individuals infected from different reservoirs had different associated risk factors: chicken consumption increased the risk for chicken-attributed infections; animal contact, barbecuing, tripe consumption, and never/seldom chicken consumption increased that for ruminant-attributed infections; game consumption and attending swimming pools increased that for environment-attributed infections; and dog ownership increased that for environment- and pet-attributed infections. Person-to-person contacts around holiday periods were risk factors for infections with exotic strains, putatively introduced by returning travellers.

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