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Sources and transmission routes of campylobacteriosis: A combined analysis of genome and exposure data

Lapo Mughini-Gras[a,b,*], Roan Pijnacker[a], Claudia Coipan[a], Annemieke C. Mulder[a], Adriana Fernandes Veludo[b], Sharona de Rijk[a], Angela H.A.M. van Hoek[a], Ralph Buij[c], Gerard Muskens[c], Miriam Koene[d], Kees Veldman[d], Birgitta Duim[e], Linda van der Graaf-van Bloois[e], Coen van der Weijden[f], Sjoerd Kuiling[a], Anjo Verbruggen[a], Joke van der Giessen[a], Marieke Opsteegh[a], Menno van der Voort[g], Greetje A.A. Castelijn[g], Franciska M. Schets[a], Hetty Blaak[a], Jaap A. Wagenaar[e], Aldert L. Zomer[e], Eelco Franz[a]

[a] Centre for Infectious Disease Control (CIb), National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
[b] Institute for Risk Assessment Sciences (IRAS), Utrecht University, Utrecht, the Netherlands
[c] Wageningen Environmental Research (WER), Wageningen University & Research (WUR), Wageningen, the Netherlands
[d] Wageningen Bioveterinary Research (WBVR), Wageningen University & Research (WUR), Lelystad, the Netherlands
[e] Department of Infectious Diseases and Immunology (I&I), Utrecht University & WHO Collaborating Center for Campylobacter/OIE Reference Laboratory for Campylobacteriosis, Utrecht, the Netherlands
[f] Netherlands Food and Consumer Product Safety Authority (NVWA), Utrecht, the Netherlands
[g] Wageningen Food Safety Research (WFSR), Wageningen University & Research (WUR), Wageningen, the Netherlands

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SUMMARY

Objectives: To determine the contributions of several animal and environmental sources of human campylobacteriosis and identify source-specific risk factors.

Methods: 1417 *Campylobacter jejuni/coli* isolates from the Netherlands in 2017–2019 were whole-genome sequenced, including isolates from human cases ($n = 280$), chickens/turkeys ($n = 238$), laying hens ($n = 56$), cattle ($n = 158$), veal calves ($n = 49$), sheep/goats ($n = 111$), pigs ($n = 110$), dogs/cats ($n = 100$), wild birds ($n = 62$), and surface water ($n = 253$). Questionnaire-based exposure data was collected. Source attribution was performed using core-genome multilocus sequence typing. Risk factors were determined on the attribution estimates.

Results: Cases were mostly attributed to chickens/turkeys (48.2%), dogs/cats (18.0%), cattle (12.1%), and surface water (8.5%). Of the associations identified, never consuming chicken, as well as frequent chicken consumption, and rarely washing hands after touching raw meat, were risk factors for chicken/turkey-attributable infections. Consuming unpasteurized milk or barbecued beef increased the risk for cattle-attributable infections. Risk factors for infections attributable to environmental sources were open water swimming, contact with dog faeces, and consuming non-chicken/turkey avian meat like game birds.

Conclusions: Poultry and cattle are the main livestock sources of campylobacteriosis, while pets and surface water are important non-livestock sources. Foodborne transmission is only partially consistent with the attributions, as frequency and alternative pathways of exposure are significant.

Introduction

Campylobacter spp. is the main reported agent of bacterial gastroenteritis worldwide, with most human campylobacteriosis cases in Europe being caused by two species: *Campylobacter jejuni* (92%) and *Campylobacter coli* (7%) (1). In the Netherlands (~17 million population), the annual number of gastroenteritis cases due to campylobacteriosis is estimated at ~70 thousand (2), with a yearly

* Corresponding author at: Centre for Infectious Disease Control (CIb), National Institute for Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, 3721MA Bilthoven, the Netherlands.
E-mail addresses: lapo.mughini.gras@rivm.nl, l.mughinigras@uu.nl (L. Mughini-Gras).

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average of 6000 reported cases. Occasionally, *Campylobacter* infection may trigger the development of sequelae beyond gastroenteritis, such as reactive arthritis, Guillain-Barré syndrome, and irritable bowel syndrome (3,4). Quantifying the relative contributions of different sources of zoonotic infections like campylobacteriosis is crucial to prioritize public health interventions.

Virtually all animals, especially avian species, may be reservoirs for *Campylobacter* and are potential sources of human infections (5). Several source attribution studies, recently reviewed by Cody et al. (6) and mainly based on conventional (seven-locus) Multilocus Sequence Typing (MLST) (7), have been conducted to quantify the sources of human campylobacteriosis. Poultry and cattle have long been identified as the main reservoirs for the *Campylobacter* strains isolated from human cases in the Netherlands, with about 60–80% of cases being attributable to chicken and 20–30% to cattle based on conventional MLST, regardless of the transmission routes involved (8,9). This is also reflected in case-control studies showing that consumption of chicken meat, as well as consumption of raw/undercooked meat (of unspecified origin), are significant risk factors for human campylobacteriosis (8,10). However, food consumption, particularly chicken meat consumption, seems to explain (as transmission route) only about half of the human campylobacteriosis cases attributable to a given food-producing animal reservoir (8,11–13). This highlights the need for further studies on *Campylobacter* transmission routes other than food, such as environment-mediated transmission.

Given the complexity of *Campylobacter* epidemiology, performing separate analyses for source attribution and risk factors is unlikely to provide full insights into the origins of human *Campylobacter* infections (8,13). Yet, combined analyses of *Campylobacter* genome and patient exposure data can bridge the gap between the attributions of human infections at the reservoir level (i.e. source attribution based on microbial subtyping) and those at the point of exposure (i.e. risk factors). Advances in high-throughput sequencing technology have made whole-genome sequencing (WGS) increasingly affordable. WGS enables unravelling of epidemiological linkages and putative transmission events among humans, animals, and the environment, proving to be a powerful tool to investigate the genomic epidemiology of microorganisms. This is particularly true for foodborne pathogens, for which WGS is increasingly becoming the standard for genotyping (14,15). Core-genome Multilocus Sequence-Typing (cgMLST) aims at enhancing the discriminatory power of conventional MLST with the extensive genomic data obtained by WGS, allowing for finer-scale differentiation of closely related bacterial strains (16–18).

In this study, we sequenced more than 1400 *C. jejuni* and *C. coli* isolates from human cases and several putative animal and environmental sources of human infections in the Netherlands. Subsequently, we applied cgMLST and performed a combined cgMLST-based source attribution and risk factor analysis to quantify the relative importance of those sources for human campylobacteriosis and unravel the underlying (source-specific) transmission routes.

Materials and methods

Human isolates

Human *C. jejuni* and *C. coli* isolates from gastroenteritis patients were obtained from 13 medical microbiology laboratories in the Netherlands collected by routine diagnostic activities between September 2017 and April 2019. Species identification was performed using Matrix-Assisted Laser-Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS, Bruker Microflex LT, Germany) at the Dutch National Institute for Public Health and the Environment (RIVM). Cases were interviewed using a comprehensive questionnaire about food consumption habits, occupation, medical history, contact with people with gastroenteritis, travel history, leisure activities, and contact with animals. The study received ethics approval from the Medical Research Ethics Committee of Utrecht University (WAG/rc/17/005968). Parents or legal guardians of minor age participants completed the questionnaire and provided informed consent on their behalf.

In total, 598 cases returned the questionnaire. After excluding the cases who traveled abroad in the seven days prior to symptom onset ($n = 170$) or without isolate available for sequencing ($n = 148$), 280 cases (272 *C. jejuni* and 8 *C. coli*) were included in the source attribution analysis. Cases who did not sign the informed consent for the analysis of questionnaires ($n = 11$) or returned an inconsistently filled in questionnaire ($n = 1$) were also excluded, resulting in 268 cases (261 *C. jejuni* and 7 *C. coli*) included in the risk factor analysis.

Animal isolates

Isolates from both faecal and meat samples of livestock animals were collected by Wageningen Bioveterinary Research (WBVR) and Wageningen Food Safety Research (WFSR), in collaboration with the RIVM and the Netherlands Food and Consumer Product Safety Authority (NVWA), within the framework of established surveillance programs for zoonotic agents, including *Campylobacter*, in food-producing animals in the Netherlands during 2014–2019. These included broiler chickens, table egg-laying hens, fattening turkeys, fattening pigs, beef and dairy cattle, veal calves, sheep and goats (i.e. small ruminants). The Veterinary Microbiological Diagnostic Centre (VMDC) of Utrecht University collected *Campylobacter* isolates from dogs and cats (i.e. pets) as part of its routine diagnostic activities on pets referred to the VMDC from veterinary clinics all over the Netherlands. Additional isolates from small ruminants were also collected for the purpose of this study, by engaging field veterinarians collaborating with the VMDC. Isolates from fresh droppings or cloacal swabs of wild birds, i.e. pigeons and common waterfowl taxa in the Netherlands, such as cormorants, gulls, geese and ducks, were collected in June and December 2018 by Wageningen Ecological Research (WER). Wild bird sampling was conducted under ethical guidelines (Art. 75 of the "Flora & Faunawet", https://wetten.overheid.nl/BWBR0009640/2016-04-14); no birds were harmed nor killed for the study.

Faecal samples were analysed without enrichment by direct streaking onto modified charcoal cefoperazone deoxycholate agar (mCCDA, Oxoid) plates in accordance with the NEN-EN-ISO 10272-1:2017 procedure. Meat samples were analyzed by use of an enrichment step in accordance with the same procedure. The isolates were identified at the species level using MALDI-TOF MS like the human isolates. In total, 186 *C. jejuni* and 14 *C. coli* isolates from broilers, 55 *C. jejuni* and 1 *C. coli* isolates from laying hens, 37 *C. jejuni* and 1 *C. coli* isolates from turkeys, 39 *C. jejuni* and 10 *C. coli* isolates from veal calves, 61 *C. jejuni* and 1 *C. coli* isolates from dairy cattle, 96 *C. jejuni* isolates from beef cattle, 86 *C. jejuni* and 25 *C. coli* isolates from small ruminants, 10 *C. jejuni* and 100 *C. coli* isolates from pigs, 95 *C. jejuni* and 5 *C. coli* isolates from pets, and 47 *C. jejuni* and 15 *C. coli* isolates from wild birds, were obtained.

Environmental isolates

In total, 90 surface water sampling sites in six geographic areas of comparable size in the Netherlands were selected based on presence of high or low density of poultry, ruminants, and pigs, according to official agricultural census data from Statistics Netherlands (www.cbs.nl). Within each of these six areas, five surface water sampling sites for each of the following three types of surface water were identified: agricultural watersheds, recreational water sites, and effluent discharge points of wastewater treatment plants

(WWTP). Each sampling site was sampled four times, once per season. Water samples were taken by submerging sterile glass bottles according to the NEN-EN-ISO 19458:2007 procedure. Samples were filtered using 0.45 μm cellulose-based membranes (Millipore) in a total volume of 1000 ml. The filters were placed in Preston broth and incubated under microaerobic conditions using CampyGen sachets (Oxoid) for 48 h at 37 °C. Samples were then streaked (10 μl) on mCCDA agar and re-incubated under microaerobic conditions for 48 h at 41.5 °C. From each sample, a maximum of five typical colonies were inspected by light microscopy for *Campylobacter* characteristics, and a maximum of five visually confirmed isolates per sample were identified at the species level using MALDI-TOF MS. In total, we obtained 253 isolates (177 *C. coli* and 76 *C. jejuni*) from surface water, considering one isolate per *Campylobacter* species from the same sample.

Sequencing

A total of 1060 *C. jejuni* and 357 *C. coli* isolates were subject to WGS (280 human, 884 animal and 253 environmental isolates). Isolation of genomic DNA was performed using the UltraClean® Microbial DNA Isolation Kit (Qiagen, USA). WGS was performed on Illumina Hiseq and NextSeq platforms (Illumina, USA) using 2×150-bp reads. Genomes were assembled with SPAdes v3.10.1 (19), checked for completeness and contamination using CheckM (20). Genomes with >5% contamination or <95% completeness were excluded. The sequences were deposited in ENA Sequence Read Archive project PRJEB38253.

A standard cgMLST scheme for *Campylobacter* population was applied as presented elsewhere (18), using Seemanns' MLST tool to scan contig files against traditional PubMLST typing schemes (https://github.com/tseemann/mlst) modified for cgMLST schemes (https://github.com/aldertzomer/cgmlst). The cgMLST profile was assessed using the sequence definitions in BIGSdb (accessed at November 9th, 2019). Additional searches of missing genes were performed using the Basic Local Alignment Search Tool (BLAST) v2.5.0 (21) on the assembled genomes. For the alleles not yet present in BIGSdb, we generated multiple alignments of each locus using MAFFT v7.407 (22) and attributed unique identification numbers. All the loci for which none of these approaches yielded an unambiguous result were considered as missing. Loci with missing allele numbers in >5% of the isolates were excluded from the analysis ($n = 88$), resulting in 1255 loci with 99.7% complete allele numbers in the whole data set.

Analysis of molecular variance

To attribute human cases to sources, genetic differentiation between the sources above the within-group heterogeneity is necessary (23). We therefore assessed the genetic heterogeneity in sequence types (STs), as derived from conventional MLST, amongst the sources by estimating Φ-statistics using analysis of molecular variance (AMOVA) (24). Sources that did not show significant mutual heterogeneity were combined into a new group. AMOVA was performed using "poppr" (version 2.8.5) and "hierfstat" (version 0.04-22) packages in R.

Source attribution analysis

The 280 isolates from human cases were attributed to the animal sources (as defined by the AMOVA) and surface water based on cgMLST data using the population genetics model STRUCTURE (version 2.3.4) (25). The model was set to specify the population of the isolates using the "USEPOPINFO" flag, and a no admixture model was used to determine the ancestry of the individuals of unknown origin, i.e. the human isolates. The length of the burn-in period was 1000 followed by 10,000 iterations. Missing allele numbers (0.3% of isolates) were handled with the default software function. For every human isolate, the model estimated a posterior (relative) probability, denoted as *Pr*, to originate from each source. These *Pr* values thereby added up to 1 over the sources for each human isolate. The attribution analysis was performed separately for *C. jejuni* and *C. coli*. However, their estimated *Pr* values were further analyzed together to obtain *a posteriori* statistics for the totality of attributed *Campylobacter* isolates, as the low number of human *C. coli* isolates ($n = 8$) did not allow for meaningful statistics at the species level. Therefore, while the analysis accounted for the different population structure of the two *Campylobacter* species, it also provided attribution and risk factor estimates that reflected the occurrence and overall epidemiology of both species in the study population. The overall proportion of human cases attributed to a given source was then calculated as the sum of its *Pr* values over cases divided by the total number of cases. 95% confidence intervals (95%CI) for the attributions were computed using bias-corrected and accelerated non-parametric bootstrapping, as implemented in Stata version 16.0 (StataCorp, College Station, USA). Previous studies (26) have shown that *Campylobacter* isolates in pets and humans are similar and that it is difficult to determine whether pets are the source of human infections or vice versa, or whether there are shared sources of infection for both pets and humans. Therefore, two source attribution analyses were performed, one including and one excluding pets as a potential source.

Risk factor analysis

Using the exposure data collected with the questionnaires, a risk factor analysis was performed. The attributions, i.e. the *Pr* values for each human case to originate from each of the sources, as estimated by STRUCTURE, were used as outcome variable to identify source-specific risk factors for human campylobacteriosis. We first performed a preliminary significance testing of 126 candidate risk factors using univariable generalized linear models (GLM) with a logit link function and binomial error distribution, which are suited to analyse proportion data (27). All analyses were adjusted for patient age (<18, 18–34, 35–64, ≥65 years), sex, degree of urbanization of residence location (urban: >2500 addresses/km^2; intermediate: 500–2500 addresses/km^2; rural: <500 addresses/km^2), season (autumn: September-November; winter: December-February; spring: March–May; summer: June–August), and highest educational level in the household (low: primary, lower vocational or lower secondary education; intermediate: intermediate vocational, intermediate secondary or higher secondary education; high: higher vocational or university education). Factors showing a p-value <0.10 for the association with the outcome in the univariable analysis were selected for inclusion in a multivariable GLM built in stepwise fashion to retain only variables with a p-value <0.05. Variables were dropped one by one only if their exclusion from the model did not change the coefficients of the other covariates by >10%. Biologically plausible interactions were also tested, and the model was expanded to include significant interaction terms, if any. Collinear variables were identified before multivariable analysis using the variance inflation factor (VIF) and selection between collinear variables was made based on improved model fit as revealed by the Akaike information criterion (AIC). The analysis was performed considering only variables with ≥5% of individuals present in each category. Risk factor analysis was performed in Stata version 16.0 (StataCorp, College Station, USA).

Table 1
Genetic heterogeneity of *Campylobacter* isolates between source populations. For each pair of sources, percent Φ values are displayed above the diagonal and the associated *p*-values below the diagonal. The higher the Φ values, the higher the differentiation between sources. Non-significant differences between sources are highlighted in bold.

	Broilers	Veal calves	Dairy cattle	Layers	Beef cattle	Pets	Small ruminants	Pigs	Turkeys	Surface water	Wild birds
Broilers		2.2%	1.4%	1.3%	2.4%	1.8%	1.7%	15.7%	**0.7%**	4.5%	4.4%
Veal calves	0.002		2.0%	5.8%	3.1%	4.4%	1.8%	13.7%	4.5%	4.9%	8.1%
Dairy cattle	0.010	0.004		3.9%	**0.9%**	3.1%	1.7%	20.0%	2.1%	5.1%	8.2%
Layers	0.020	0.001	0.001		3.6%	1.9%	2.4%	18.8%	1.8%	3.0%	6.1%
Beef cattle	0.001	0.002	**0.100**	0.001		2.4%	1.5%	20.7%	1.9%	5.1%	7.4%
Pets	0.010	0.001	0.002	0.003	0.001		1.9%	18.8%	1.5%	2.8%	5.4%
Small ruminants	0.003	0.010	0.010	0.003	0.020	0.004		14.0%	1.6%	3.5%	5.7%
Pigs	0.001	0.001	0.001	0.001	0.001	0.001	0.001		17.9%	14.4%	16.5%
Turkeys	**0.317**	0.001	0.004	0.010	0.010	0.030	0.020	0.001		2.8%	6.9%
Surface water	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001		2.1%
Wild birds	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.010	

Results

Source heterogeneity

Table 1 shows the Φ-values and corresponding p-values for each pair of sources from the AMOVA. There was significant heterogeneity between most of the sources. The only non-significant heterogeneities were observed between broilers and turkeys, and between dairy and beef cattle. Therefore, these sources were combined into 'meat-producing poultry' (i.e. broilers and turkeys) and 'adult cattle' (i.e. dairy and beef cattle) for further analyses.

Genotype distribution

Overall, the 1060 *C. jejuni* and 357 *C. coli* isolates were respectively assigned to 189 and 77 known seven-locus STs, whereas 73 and 188 isolates belonged to novel STs. The most frequent STs were ST-21, ST-45, ST-48, ST-19, ST-6175, and ST-42, all belonging to *C. jejuni* and representing almost a quarter of all isolates with a known ST. ST-21, ST-19, and ST-6175 belong to the same clonal complex (CC): CC-21. Overall, ST-21 and ST-45 were widely distributed over the sources, although mainly represented in ruminants (ST-21), pets and surface water (ST-45) (Supplementary Table S1). ST-48, ST-19, ST-6175 and ST-42 were less widespread and were mostly present in meat-producing poultry (ST-6175), adult cattle (ST-42), or both (ST-19 and ST-48), as well as humans (ST-19). Of the 280 human isolates, only 13 (4.6%) isolates were not assigned to a known ST, while the majority of isolates belonged to 85 different STs. The most predominant STs among human isolates were ST-21, ST-6175, ST-50, ST-19 and ST-52, representing over a quarter of all human isolates, all belonging to *C. jejuni*. Besides humans, ST-50 was mainly found in meat-producing poultry, while ST-52 was rare among non-human isolates.

The population structures of *C. jejuni* and *C. coli* isolates are visualized respectively in Figs. 1 and 2 using a minimum-spanning tree (MST) based on cgMLST. Each circle represents a different cgMLST type and the size of the circles is proportional to the number of isolates of that specific type, while the colors indicate the different sources in which that type was found. The human *C. jejuni* isolates were distributed along the MST, but predominantly in clusters dominated by meat-producing poultry isolates. The few human *C. coli* isolates clustered mainly with meat-producing poultry, small ruminant and surface water isolates.

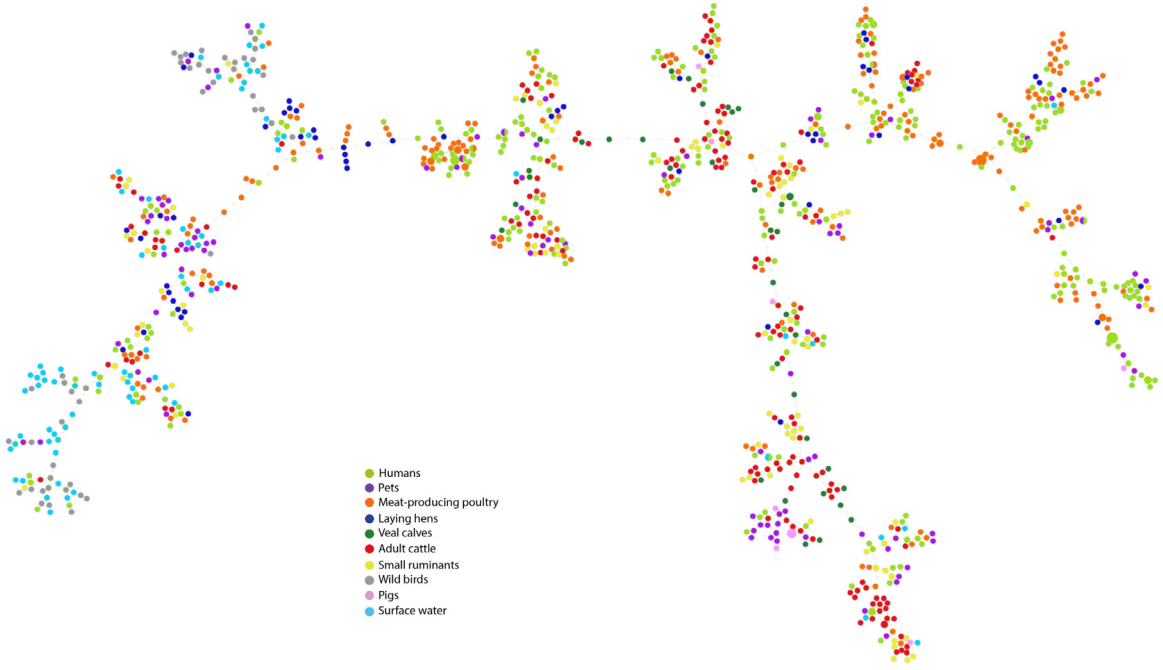

Fig. 1. Minimum spanning trees for *Campylobacter jejuni* isolates in human patients, animal and environmental sources based on cgMLST. The size of the circles represents the number of times a given type has been found and the colors indicate the different sources in which that type has been found.

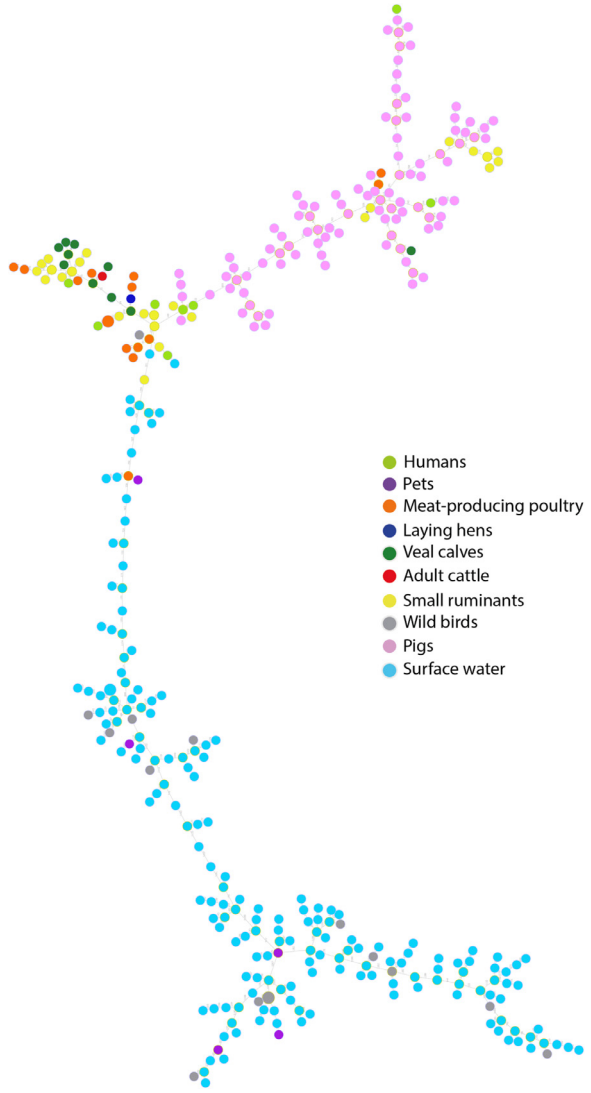

Fig. 2. Minimum spanning trees for *Campylobacter coli* isolates in human patients, animal and environmental sources based on cgMLST. The size of the circles represents the number of times a given type has been found and the colors indicate the different sources in which that type has been found.

Attributable sources

cgMLST was used to quantify the attributable animal and environmental sources of the 280 human isolates. In the analysis including also pets as a potential source, 48.2% (95%CI 42.7–53.6%) of all human isolates were attributed to meat-producing poultry, followed by pets (18.0%, 95%CI 13.7–22.2%), adult cattle (12.1%, 95%CI 8.9–15.3%), surface water (8.5%, 95%CI 5.5–11.4%), small ruminants (7.4%, 95%CI 4.8–10.1%), laying hens (3.2%, 95%CI 1.2–5.2%), veal calves (2.2%, 95%CI 0.8–3.5%), wild birds (0.4%, 95%CI 0.0–1.0%), and pigs (0.1%, 95%CI 0.0–0.2%) (Fig. 3). When pets were excluded from the analysis, the human cases were attributed as follows: meat-producing poultry (60.3%, 95%CI 54.8–65.8%), adult cattle (13.3%, 95%CI 9.9–16.7%), surface water (11.2%, 95%CI 7.8–14.5%), small ruminants (8.7%, 95%CI 5.8–11.6%), laying hens (3.5%, 95%CI 1.5–5.6%), veal calves (2.6%, 95%CI 1.0–4.1%), wild birds (0.4%, 95%CI 0.0–1.0%), and pigs (0.1%, 95%CI 0.0–0.2%) (Fig. 3).

Risk factors

General demographics of the 598 human cases who returned the questionnaire are summarized in Table 2. The most represented age groups were those aged 35–64 (39%) and ≥65 (29%) years. Cases were evenly distributed between males (52%) and females (48%). Most cases reported diarrhoea (96%), stomach-ache (90%), nausea (61%), and fever (59%), followed by mucus in the stool (47%), blood in the stool (30%), and vomiting (27%). Mean duration of illness was 14 days (95%CI 12–16), with 21% of cases reporting to have been hospitalized for an average of 4.5 days (95%CI 4.0–5.0).

Table 2
General demographics of the human campylobacteriosis cases returning the epidemiological questionnaire.

	Number of cases (and percentage) returning the questionnaire	Number of cases (and percentage) enrolled in the risk factor analysis
Total	598	268
Sex		
Female	281 (46.9)	118 (44.0)
Male	307 (51.3)	146 (54.5)
Unknown	10 (1.7)	4 (1.5)
Age (years)		
<18	53 (8.9)	23 (8.6)
18–34	134 (22.4)	52 (19.4)
35–64	227 (38.0)	97 (36.2)
≥65	170 (28.4)	93 (34.7)
Unknown	14 (2.3)	3 (1.1)
Educational level[a]		
Low	137 (22.9)	75 (28.0)
Middle	233 (39.0)	107 (39.9)
High	189 (31.6)	64 (23.9)
Unknown	39 (6.5)	22 (8.2)
Degree of urbanization[b]		
Urban	122 (20.4)	45 (16.8)
Intermediate	340 (56.9)	149 (55.6)
Rural	135 (22.6)	67 (25.0)
Unknown	1 (0.2)	7 (2.6)
Season[c]		
Winter	97 (16.2)	49 (18.3)
Autumn	159 (26.6)	71 (26.5)
Spring	109 (18.2)	59 (22.01)
Summer	209 (34.9)	80 (29.9)
Unknown	24 (4.0)	9 (3.4)

[1a] Low: primary, lower vocational or lower secondary education; intermediate: intermediate vocational, intermediate secondary or higher secondary education; high: higher vocational or university education.
[2b] urban: >2500 addresses/km^2; intermediate: 500–2500 addresses/km^2; rural: <500 addresses/km^2. [c]autumn: September–November; winter: December-February; spring: March–May; summer: June–August.

Source-specific risk factors for the 268 campylobacteriosis cases could be studied for meat-producing poultry, adult cattle, environmental sources (i.e. surface water and wild birds combined), pets, small ruminants, and laying hens, but not for veal calves and pigs due to the very low attributions for these sources. Seven factors were significantly associated with infections with *Campylobacter* strains originating from meat-producing poultry in the final multivariable model (Table 3). Never consuming chicken meat, as well as frequent (i.e. weekly/daily) consumption of chicken meat, were significantly associated with increased probabilities (i.e. attributions) for the infecting strains to originate from meat-producing poultry, as compared to monthly consumption of chicken meat. Other risk factors were rarely washing hands after handling raw meat (and before touching other foods), having consumed lamb/mutton or having had contact with cat faeces in the seven days prior to symptom onset, and having had contact household members with gastroenteritis. Factors significantly associated with decreased probabilities for the infecting strains to originate from meat-producing poultry were having several children aged 0–11 years living in the household and having traveled (with overnight stay) within the Netherlands in the seven days prior to symptom onset.

Four factors were significantly associated with increased probabilities for the infecting strains to originate from adult cattle

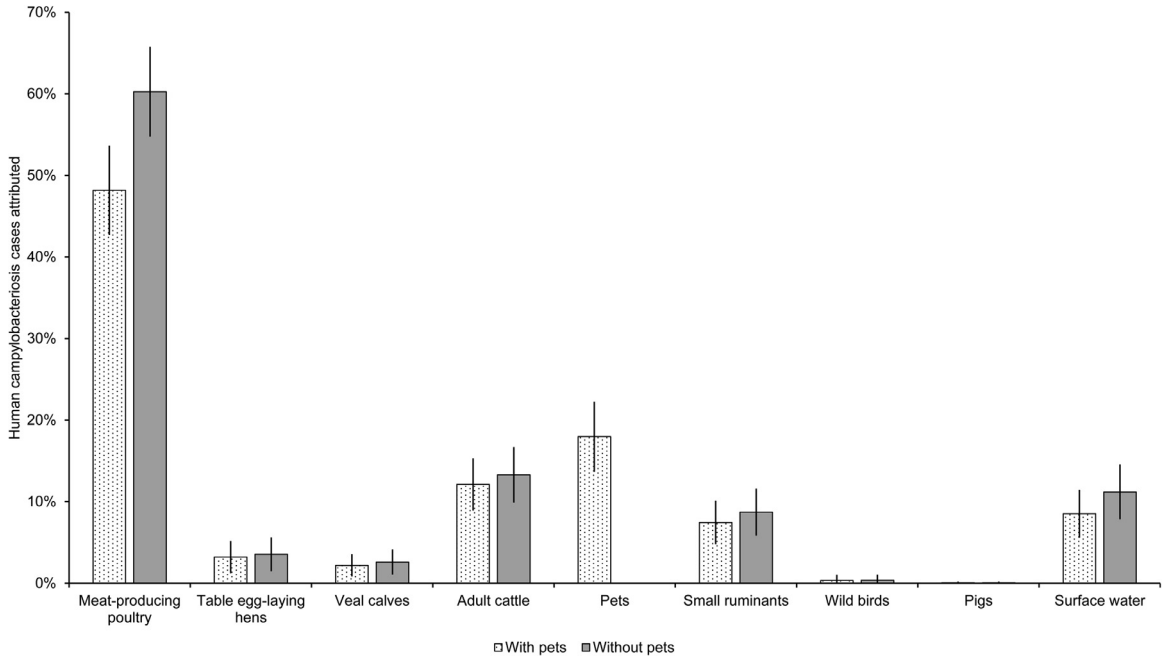

Fig. 3. Estimated fractions of human campylobacteriosis cases ($n = 280$) attributed to the animal and environmental sources, including and excluding pets as a potential source. Error bars denote 95% confidence intervals.

Table 3
Factors significantly associated with human *Campylobacter* infections attributable to meat-producing poultry.

Risk factor	β-coefficient[a]	95% confidence interval		p-value
Number of children aged 0–11 years living in the household (continuous)	−0.642	−1.140	−0.143	0.012
Domestic travel (within the Netherlands) with overnight stay in the 7 days prior to symptom onset (y/n)	−1.081	−1.846	−0.316	0.006
Frequency of chicken meat consumption				
Never	0.895	0.008	1.782	0.048
Less than monthly	0.481	−0.984	1.946	0.520
Monthly	Reference			
Weekly or daily	1.051	0.315	1.787	0.005
Washing hands after handling raw meat (and before touching other foods)				
Always	Reference			
Sometimes	0.435	−0.455	1.324	0.338
Rarely	1.716	0.184	3.247	0.028
Consumption of lamb/mutton in the 7 days prior to symptom onset (y/n)	0.939	0.114	1.763	0.026
Contact with household members with gastroenteritis (y/n)	1.368	0.438	2.299	0.004
Contact with cat faeces in the 7 days prior to symptom onset (y/n)	1.182	0.020	2.345	0.046

[1a] Estimates are adjusted for age, sex, urbanization degree of residence location, season, and highest educational level in the household (see Table 1 for details about these variables), besides the factors shown in the table. y/n = binary 'yes or no' variable.

(Table 4). These were consumption of unpasteurized milk or consumption of barbecued beef in the seven days prior to symptom onset, consumption of dairy products other than milk or cheese on a monthly or more frequent basis (vs. never consuming these products), and having traveled (with overnight stay) within the Netherlands in the seven days prior to symptom onset. Conversely, suffering from diabetes, being employed in childcare, and consumption of raw eggs or raw egg-containing products in the seven days prior to symptom onset were significantly associated with decreased probabilities for the infecting strains to originate from adult cattle.

Three factors were significantly associated with increased probabilities for the infecting strains to originate from the environmental sources (i.e. surface water and wild birds) (Table 5). These were having swum in open waters, having had contact with dog faeces, or having consumed meat of avian species other than chickens and turkeys (i.e. ducks, geese, quails, pheasants and game bird meat) in the seven days prior to symptom onset. Conversely, having a respiratory comorbidity was a protective factor.

Three factors were significantly associated with increased probabilities for the infecting strains to originate from pets (Table 6). These were owning one or more dogs and/or cats in households with children aged 0–11 years (vs. no dogs nor cats owned at all), consuming chicken meat monthly or more frequently (vs. never or hardly ever consuming chicken meat), and having consumed liver pâté in the seven days prior to symptom onset, whereas consuming pork monthly or more frequently (vs. never or hardly ever consuming pork) was a protective factor.

Three factors were significantly associated with increased probabilities for the infecting strains to originate from small ruminants (Table 7). These were consumption of meat salad or consumption of meat in pastry in the seven days prior to symptom onset, whereas consumption of chicken meat in the seven days prior to symptom onset was a protective factor.

Consumption of meat substitutes in the seven days prior to symptom onset was the only factor significantly associated with increased probabilities for the infecting strains to originate from table egg-laying hens (Table 8). Conversely, consumption of pork

Table 4
Factors significantly associated with human *Campylobacter* infections attributable to adult cattle.

Risk factor	β-coefficient[a]	95% confidence interval		*p*-value
Occupation in childcare (y/n)	−3.889	−6.080	−1.697	0.001
Suffering from diabetes (y/n)	−1.457	−2.821	−0.093	0.036
Domestic travel (within the Netherlands) with overnight stay in the 7 days prior to symptom onset (y/n)	0.972	0.096	1.848	0.030
Consumption of raw egg (products) in the 7 days prior to symptom onset (y/n)	−3.456	−6.087	−0.824	0.010
Consumption of unpasteurized milk in the 7 days prior to symptom onset (y/n)	1.410	0.185	2.634	0.024
Frequency of consumption of dairy products other than milk and cheese				
Never	Reference			
Less than monthly	1.213	−0.823	3.250	0.243
Monthly	2.089	0.402	3.776	0.015
Weekly or daily	1.988	0.507	3.468	0.008
Consumption of beef and barbecued meat in the 7 days prior to symptom onset	1.128	0.147	2.109	0.024
No beef nor barbecued meat consumed	Reference			
Consumed non-barbecued beef	0.470	−1.154	2.110	0.566
Consumed barbecued meat (albeit no beef)	0.201	−1.916	2.317	0.853
Consumed barbecued beef	1.692	0.181	3.202	0.028

[1a] Estimates are adjusted for age, sex, urbanization degree of residence location, season, and highest educational level in the household (see Table 1 for details about these variables), besides the factors shown in the table. y/n = binary 'yes or no' variable.

Table 5
Factors significantly associated with human *Campylobacter* infections attributable to the environmental sources (i.e. surface water and wild birds).

Risk factor	β-coefficient[a]	95% confidence interval		*p*-value
Suffering from a respiratory comorbidity (y/n)	−0.520	−0.970	−0.070	0.024
Swimming in open waters in the 7 days prior to symptom onset (y/n)	0.470	0.020	0.919	0.040
Contact with dog faeces in the 7 days prior to symptom onset (y/n)	0.685	0.141	1.229	0.014
Consumption of meat of avian species other than chickens or turkeys (i.e. ducks, geese, quails, pheasants or game bird meat) in the 7 days prior to symptom onset (y/n)	0.405	0.010	0.799	0.045

[1a] Estimates are adjusted for age, sex, urbanization degree of residence location, season, and highest educational level in the household (see Table 1 for details about these variables), besides the factors shown in the table. y/n = binary 'yes or no' variable.

Table 6
Factors significantly associated with human *Campylobacter* infections attributable to pets (i.e. dogs and cats).

Risk factor	β-coefficient[a]	95% confidence interval		p-value
Ownership of dogs and/or cats	0.847	0.092	1.603	0.028
No dogs nor cats owned	Reference			
One or more dogs and/or cats owned in a household without children aged 0–11 years	−0.476	−1.306	0.354	0.261
One or more dogs and/or cats owned in a household with children aged 0–11 years	1.389	0.209	2.571	0.021
Frequency of chicken meat consumption (less than monthly vs. monthly or more often)	1.306	0.339	2.273	0.008
Frequency of pork consumption (less than monthly vs. monthly or more often)	−1.065	−1.917	−0.214	0.014
Consumption of liver pâté in the seven days prior to symptom onset (y/n)	0.950	0.159	1.740	0.019

[1a] Estimates are adjusted for age, sex, urbanization degree of residence location, season, and highest educational level in the household (see Table 1 for details about these variables), besides the factors shown in the table. y/n = binary 'yes or no' variable.

Table 7
Factors significantly associated with human *Campylobacter* infections attributable to small ruminants (i.e. sheep and goats).

Risk factor	β-coefficient[a]	95% confidence interval		p-value
Number of children aged 12–17 years living in the household (continuous)	−1.807	−3.079	−0.536	0.005
Consumption of meat salad in the 7 days prior to symptom onset (y/n)	1.387	0.299	2.475	0.012
Consumption of meat in pastry in the 7 days prior to symptom onset (y/n)	1.472	0.487	2.457	0.003
Consumption of chicken meat in the 7 days prior to symptom onset (y/n)	−1.766	−2.788	−0.744	0.010

[1a] Estimates are adjusted for age, sex, urbanization degree of residence location, season, and highest educational level in the household (see Table 1 for details about these variables), besides the factors shown in the table. y/n = binary 'yes or no' variable.

Table 8
Factors significantly associated with human *Campylobacter* infections attributable to table egg-laying hens.

Risk factor	β-coefficient[a]	95% confidence interval		p-value
Consumption of meat substitutes in the 7 days prior to symptom onset (y/n)	2.634	0.612	4.656	0.011
Consumption of pork in the 7 days prior to symptom onset (y/n)	−2.937	−5.292	−0.581	0.015

[1a] Estimates are adjusted for age, sex, urbanization degree of residence location, season, and highest educational level in the household (see Table 1 for details about these variables), besides the factors shown in the table. y/n = binary 'yes or no' variable.

in the seven days prior to symptom onset was a protective factor.

Discussion

This is the first combined analysis of cgMLST-based source attribution and case exposure data to quantify the sources of human campylobacteriosis and to identify source-specific risk factors. Previous studies were based on conventional MLST. Moreover, either a source-assigned case-control (8,13,28) or case-case (29–31) study was conducted. In those studies, groups of cases were first assigned to specific sources based on their attributions and then the exposures of these groups of cases were compared with one another or with those of a control group (32). Here instead, we

modelled the attributions directly with the corresponding exposure data for cases only.

Strain diversity, as depicted by seven-locus STs, was substantial, with 1156 isolates belonging to 266 different STs. There were more isolates with novel STs among *C. coli* than *C. jejuni* isolates, which is likely due to *C. coli* isolates being commonly found in surface water and wild birds. In previous studies, water- and wild bird-associated isolates have been under-represented relative to isolates from humans and domesticated animals, which may explain the higher occurrence of novel STs in those sources. Although surface water cannot be considered as a reservoir or 'amplifying host' for *Campylobacter*, it represents a 'sink' that collects strains from a variety of different hosts, including those found in animals and humans (9,13,33,34). Therefore, as pointed out elsewhere (8,9,13,33,34), surface water can also be considered as a proxy for other unidentified (animal) reservoirs, including wildlife. ST-21 was the predominant ST in humans, followed by ST-6175, ST-50, ST-19, and ST-52, which all have been previously reported among human cases in several European countries (7,13,28,35–38), including the Netherlands (8,9,26). Previous studies reported ST-21 to be particularly prevalent in cattle and poultry (7,35–40), with some reports from sheep as well (40). The findings in this study are consistent with previous observations, as ST-21 was found to occur frequently among ruminant isolates. ST-19, one of the other predominant STs among human isolates, has also been reported to be prevalent in cattle (36) and poultry (35,39), as confirmed in this study. Also similar to previous studies (7,8), it was observed that ST-6175 and ST-50 were highly prevalent in poultry, with no or little occurrence in ruminants. ST-6175 is a poorly documented ST in the literature, with only a few reports from poultry (41), while it was prevalent in meat-producing poultry here. ST-52 was mainly prevalent in humans, with only a few isolates from animals, as observed previously (36,37).

Meat-producing poultry, i.e. broilers and turkeys, was confirmed again to be the primary source of human campylobacteriosis in the Netherlands, accounting for about half of the cases. The second most important livestock source was adult cattle, accounting for 12–13% of cases (and up to 21% of cases when considering ruminants altogether, i.e. adult cattle, veal calves, sheep and goats). This is in line with previous studies in the Netherlands (8,9) and other industrialized countries (13,23,28,34,36,42), although ruminants have recently been reported to be the primary source of human campylobacteriosis in France (43), especially for non-invasive *Campylobacter* infections (44). The inclusion of pets in the source attribution analysis revealed that they were a sizeable source, with about 18% of human cases attributed to pets, which is higher than previous attributions from Switzerland (9%) (45), France (12%) (43), and Germany (14%) (28), but lower than in a previous Dutch study (25%) (26). The epidemiological role of pets in *Campylobacter* transmission to humans is unclear, as humans and their pets often share their living environments in the household and the transmission may therefore also occur from owners to pets. Moreover, while ownership of dogs, particularly puppies, has been reported to be a significant risk factor for human campylobacteriosis (8,26), it is also possible that pets acquire *Campylobacter* carriage in parallel with humans from a common source (26). This is mainly because pet foods and treats, which are handled by pet owners, contain ingredients of the same animal origins as the food consumed by humans. Furthermore, pets are often fed with the same foods as their owners when they are offered a homemade diet or kitchen food scraps, especially raw meats, offal, and bones, the consumption of which is a risk factor for *Campylobacter* carriage in pets (46). As the source attribution analysis was non-directional in the transmission of infection, our results provided evidence for a substantial association of *Campylobacter* strains between humans and pets, but cannot provide evidence as to whether and how transmission of such strains occurred. It follows, therefore, that the attributions for pets might be an overestimation, as we cannot fully exclude that the model attributed isolates to pets instead of the common reservoirs for pets and humans. When excluding pets from the model, cases attributable to meat-producing poultry increased considerably (+12%), followed by cases attributed to the environmental sources (+3%), whereas the other sources remained almost invariant. These differences are suggestive of the sources from which pets might acquire *Campylobacter* infection in parallel with humans (26). This hypothesis was also supported by the risk factor analysis, as contact with cat faeces was associated with infections attributable with meat-producing poultry, and frequent chicken meat consumption and consumption of liver pâté (which is often made of chicken liver) were associated with infections caused by pet-attributable *Campylobacter* strains. Moreover, contact with dog faeces was associated with infections with strains attributable to the environmental sources. These associations further suggest that those sources and exposures are interconnected. Nonetheless, the role of pets remains unclear, as besides dog/cat ownership, the other risk factors had no straightforward mechanistic interpretation.

Surface water appeared to be a sizeable source, accounting for up to 11% of human cases, which is in agreement with the attributions of 10% or less reported in previous Dutch studies (8,9). As mentioned before, surface water is not *per se* a reservoir for *Campylobacter,* but a collection vessel of strains from multiple hosts. The observed attribution of water may therefore also at least partially reflect attributions to 'other sources' contaminating surface water that were not explicitly included in the analysis. In this regard, quantifying the sources of surface water contamination with *Campylobacter* might be insightful. A study in Luxemburg and the Netherlands found that most *Campylobacter* strains in surface water were attributable to wild birds and poultry, indicating significant contamination with (wild) animal faeces and agricultural effluents (47). This provided insights into the potential role of the environment concerning numerous human campylobacteriosis cases that cannot be epidemiologically explained by foodborne transmission alone (48). Similar conclusions were also reached by a New Zealand study on *C. jejuni* strains associated with wild birds and those causing human disease in six high-use recreational waterways (49).

While the source attribution analysis quantified the relative contributions of the different sources to the human cases, the risk factor analysis identified factors associated with infection with *Campylobacter* strains attributable to specific sources. This allowed for the identification of possible pathways by which these strains might have reached and infected humans from their original sources. We found that either frequently or never consuming chicken meat were associated with infection with strains attributable to meat-producing poultry. Chicken meat consumption has long been identified as the main risk factor for human campylobacteriosis, including infections attributable specifically to the chicken reservoir (8,13,28). Yet, this association may be nuanced with regard to the frequency of chicken meat consumption and acquisition of immunity. Indeed, it might be that people who frequently consume chicken meat are highly exposed to chicken-associated *Campylobacter* strains and therefore are at increased risk of acquiring the infection and falling ill with these strains. Conversely, people who do not usually include chicken meat in their diet would hardly ever be exposed to these strains and are therefore unable to develop any immunity against them, thereby falling ill more easily upon (incidental) exposure to them, which does not necessarily have to occur via food. This hypothesis entails that with a weekly/daily consumption of chicken meat, the level of exposure might be too high to allow acquired immunity to exert a protective effect of any kind. Previous studies found that repeated ex-

posure to *Campylobacter* may lead to sufficient immunity to provide some protection against severe clinical symptoms, but not illness (campylobacteriosis) *per se* (50–52). It has also been shown that consumption of chicken meat is a risk factor only or predominantly when this is consumed outside the household (13,52–54). This suggests an effect of exposure to chicken-associated *Campylobacter* strains beyond domestic food handling and consumption due to increased chance (outside the home) of being exposed to (higher doses of) specific *Campylobacter* strains different from those to which people are (usually) exposed at home (13,52,53). However whether such (temporary and limited) acquired immunity is able to outweigh the associated disease burden of human campylobacteriosis, both in terms of frequent mild illness and the less frequently occurring sequalae, remains unclear. For infections attributable to meat-producing poultry, we also found that rarely washing hands after handling raw meat was a significant risk factor. This highlights the importance of cross-contamination in the kitchen, which is particularly important for *Campylobacter* transmission from poultry meat, as this meat is usually consumed thoroughly cooked in contrast to, e.g., beef, which is often purposely consumed raw/undercooked (55). 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* (56).

Consumption of unpasteurized milk, as well as frequent consumption of dairy products other than milk or cheese, and consumption of barbecued beef, were associated with infection with strains attributable to adult cattle. A study in New Zealand has also found that human infections with *Campylobacter* strains attributable to cattle were significantly associated with raw milk consumption (54). Despite the relatively high carriage of *Campylobacter* in cattle (57), there is only little evidence that consumption of beef is an important risk factor for human campylobacteriosis in general (8,13). Indeed, beef is rarely contaminated with *Campylobacter*, and where contamination exists, it is usually at low concentrations (58). Yet, a significant association between barbecued meat consumption and infection with *Campylobacter* strains of cattle origin has been reported before (8). An explanation is that red meats in general, and particularly beef, is highly 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 (8). On the other hand, several campylobacteriosis outbreaks have been linked to consumption of unpasteurized milk, e.g. (59,60). Although we did not have specific information regarding the dairy products other than milk or cheese, the frequency of consumption of these unidentified products appeared to pose a risk of infection related to increased exposure to the pathogen. Moreover, consumption of other types of protein sources (i.e. eggs) appeared to be protective against infection with cattle-associated strains, and so was consumption of chicken meat for infection with small ruminant-associated strains and consumption of pork for infection with laying hen-associated strains. These negative associations support the hypothesis that people consuming these products could be less at risk of infection with strains originating from other sources, as speculated previously (8).

For infections attributable to laying hens, although commercial eggs are unlikely to pose a public health risk for campylobacteriosis (61), as *Campylobacter* does not colonize the avian female reproductive tract, the few significant risk factors appeared to be related to a 'meatless' diet (e.g. vegetarian meat substitutes). Meat seemed to play a direct role for infections with small ruminant-associated strains, with consumption of 'meat salad' and 'meat in pastry' being significant. In general, however, it is puzzling to interpret some of the significant associations we found, such as the effects of occupation, household composition, and comorbidities, which possibly reflect some hitherto unknown exposures linked to activities, hygiene practices, and eating habits more typical of certain groups of the population. On the other hand, factors associated with infection with strains attributable to the environmental sources were plausible and in line with previous studies (8,13). Indeed, swimming in surface water and consuming meat of avian species other than chickens and turkeys, such as ducks, geese, quails, pheasants and game bird meat, were significant risk factors, which is consistent with the environmental sources including both surface water and wild birds. As *Campylobacter* is widespread in surface water (47), the risk posed by swimming in particular was anticipated. Also the significant association with contact with dog faeces is plausible, as dogs with outdoor access may act as vectors for environmental strains (8,26), especially if they have access to fields grazed by livestock or wildlife (62). Furthermore, owners may be particularly exposed to these environmental strains themselves while walking their dogs outdoor.

This study has some limitations. Firstly, the risk factor analysis included only case exposure data. Although this study design eliminated issues related to, e.g. differential recall bias, selection bias, misclassification, etc. between cases and controls, it is important to note that the risk factors identified here were derived from (finer-scale) differences in attributions amongst the cases themselves and not from the comparison of exposures between (source-assigned) cases and a common control group. Yet, this approach also had the advantage to better pinpoint the source-specific risk factors by filtering out those factors that are common to most, if not all, cases, such as some underlying diseases, use of certain medicines like gastric antacids, factors related to unhealthy lifestyles, etc. which have previously been found to be universal risk factors for campylobacteriosis regardless of the attributable sources in question (8,13,28). Other limitations were related to different isolation media, sample size and multiple hypothesis testing. However, this study was explorative in nature and meant to generate, rather than conclusively test, hypotheses that will benefit from a closer look in more specific studies. Finally, as cases originated from routine diagnostic activities of people with gastroenteritis seeking medical care, they represent the most severe, symptomatic infections occurring in the population. Thus, the attributions and source-specific risk factors identified here pertained to severe campylobacteriosis and might differ when considering the whole spectrum of the infection. However, serological studies have indicated that factors associated with increased exposure to *Campylobacter* are similar to those associated with increased risk of clinically overt campylobacteriosis (63).

In conclusion, this study bridged the gap of exploring risk factors for human campylobacteriosis at the point of exposure while accounting for the likely origins of the infecting *Campylobacter* strains, using a combined source attribution (based on high-resolution genomic data) and case exposure analysis. With this approach, we confirmed that meat-producing poultry and cattle are the main livestock reservoirs of human campylobacteriosis, and that pets and surface water are important non-livestock sources. The attributions to livestock sources were only partially consistent with foodborne transmission, as significant effects of frequency and alternative pathways of exposure were observed as well. Overall, we showed that risk factors for *Campylobacter* infection differ depending on the attributable reservoirs and that a joint analysis of core genome and epidemiological data may provide novel insights into the origins and transmission pathways of human campylobacteriosis.

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Declaration of Competing Interest

The authors have no competing interests to declare.

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