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Campylobacter Immunity and Coinfection following a Large Outbreak in a Farming Community▽†

K. J. Forbes,[1] F. J. Gormley,[1] J. F. Dallas,[1] O. Labovitiadi,[1] M. MacRae,[1] R. J. Owen,[2] J. Richardson,[2] N. J. C. Strachan,[3] J. M. Cowden,[4] I. D. Ogden,[1]* and C. C. McGuigan[5]

Department of Medical Microbiology, School of Medicine, University of Aberdeen, Aberdeen,[1] Health Protection Agency, Centre for Infections, London,[2] School of Biological Sciences, University of Aberdeen, Aberdeen,[3] Health Protection Scotland, Clifton House, Clifton Place, Glasgow,[4] and NHS Tayside Health Protection Team, Kings Cross Hospital, Dundee,[5] United Kingdom

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An outbreak of campylobacteriosis affected approximately one-half of 165 people attending an annual farmers' dance in Montrose, Scotland, in November 2005. Epidemiological investigations, including a cohort study ($n = 164$), identified chicken liver paté as the most likely vehicle of infection. Paté preparation involved deliberate undercooking of chicken livers by flash-frying, followed by mechanical homogenization. Typing of 32 *Campylobacter* strains (isolated from submitted stools) by multilocus sequence typing identified four distinct clades of *Campylobacter jejuni*. There was good agreement when isolates were typed by Penner serotyping, pulsed-field gel electrophoresis, and *flaA* short variable region sequencing but poorer agreement with phage and antibiotic susceptibility testing. At least three attendees were coinfected with two *Campylobacter* strains each. The outbreak was probably due to several livers contributing *Campylobacter* strains that survived undercooking and were dispersed throughout the paté. The study highlights improper culinary procedures as a potential human health risk and provides a striking counterexample to the "dominant outbreak strain" view of point source outbreaks of food-borne infections. It also demonstrates that previous exposure to biologically plausible sources of *Campylobacter* may confer protection against subsequent infection.

Developed countries allocate considerable resources to assessing the human health burden of food-borne disease (16). Detecting disease outbreaks plays an increasingly important part in public health sectors, and detection and strain comparison are increasingly dependent on molecular subtyping of pathogens. A single, dominant strain is often viewed as the cause of a point source outbreak (40) and can underpin large-scale surveillance programs for monitoring outbreaks of pathogens such as *Salmonella*, *Listeria*, and *Escherichia coli* O157 (49). Whether the "outbreak strain" assumption is always applicable to point source outbreaks of other food-borne pathogens, however, is unclear.

Campylobacter infection is the commonest reported cause of food-borne gastroenteritis in developed countries, and almost all cases in the United Kingdom are due to infection from two species, with *Campylobacter jejuni* accounting for approximately 90% of cases and *Campylobacter coli* accounting for most of the remainder (39). The epidemiology of human *Campylobacter* infection is complex, with food (1), water (45), and environmental sources (18) all contributing. Specifically, the handling of raw poultry and eating undercooked chicken carry high relative risks of *Campylobacter* infection (1). The majority of clinical cases present as sporadic, with nonhousehold outbreaks rarely being identified. Attempts to trace routes of *Campylobacter* infection are therefore based mostly on case-control studies, although point source outbreaks might be commoner than current epidemiological surveillance would suggest (9) and may play a key role in mitigation strategies.

A number of phenotypic and molecular typing methods are used to identify outbreak-associated *Campylobacter* strains in specific food-borne or waterborne outbreaks (10, 15, 42, 43, 46). Matching patient isolates from outbreaks to those from potential vehicles of infection can help to implicate them and can suggest sources and causes of infection (32, 47). The "outbreak strain" view might nonetheless be simplistic for some point source food-borne outbreaks of campylobacteriosis. Broiler flocks are often coinfected with *Campylobacter* strains of different genotypes (27, 28, 34). Cross-contamination of strains among birds during slaughter (41) or subsequent food processing potentially provides further opportunities for strain mixing. Chicken meat and animal livers for retail sale in the United Kingdom are often contaminated with more than one *Campylobacter* strain (31). Coinfection of humans with more than one *Campylobacter* strain is identified in 5 to 10% of apparently sporadic cases (44) and approximately one-half of outbreaks (20) of campylobacteriosis in the United Kingdom.

There is some evidence of the occurrence of acquired immunity to campylobacteriosis, and such evidence often relates to animals (4, 5, 36, 50). For humans, however, there are ethical and financial difficulties with large-scale controlled trials involving deliberate exposure. The large size of the outbreak reported here and the fact that a number of members of the cohort (farmers and veterinarians) had previously been exposed to a biologically plausible source of *Campylobacter* (animals) allowed the study of differential attack rates between subgroups who were and were not previously exposed.

This study focuses on an outbreak of human campylobacte-

* Corresponding author. Mailing address: School of Medicine, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, United Kingdom. Phone: 44 1224 551132. Fax: 44 1224 552692. E-mail: i.ogden@abdn.ac.uk.

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TABLE 1. Animal occupation association of attendees who ate paté

Preexposure group	No. of individuals reporting diarrhea (*P* value)	Relative risk	No. of individuals with *Campylobacter*	Total no. of individuals (*P* value)	Relative risk
Animal worker	17 (0.025)	0.679	8	33 (0.29)	0.766 (0.386–1.52)
Non-animal worker	51	1	25	69	1
Total	68		33[a]	102	

[a] One sample was unavailable for typing.

riosis following an annual farmers' dinner dance held in Montrose, Scotland, in November 2005. The buffet dinner allowed a choice of main courses and portion sizes. The first course alternatives included chicken liver paté, and chicken was also a popular choice in the main course. The goals of this study were (i) to identify the vehicle, source, and cause of infection; (ii) to quantify the diversity in the *Campylobacter* strains involved; (iii) to reassess the outbreak strain view of point source campylobacteriosis outbreaks; and (iv) to compare relative risks of acquiring infection for those who worked directly with animals with those for persons who did not.

MATERIALS AND METHODS

Outbreak epidemiology. On 28 November 2005, an environmental health officer at Angus Council contacted the NHS Tayside Health Protection Team to say that 15 people who had attended a farmers' dance on 25 November had reported having diarrhea and/or vomiting symptoms. The health protection team first met on 30 November 2005, when the following case definitions were determined: (i) possible case, a person who attended the farmers' dance and developed symptoms consistent with gastroenteritis between noon on 26 November 2005 and noon on 3 December 2005; (ii) clinical (probable) case, a possible case with diarrhea (two or more loose bowel movements) between noon on 26 November 2005 and noon on 3 December 2005; and (iii) microbiological (confirmed) case, a possible case where *Campylobacter* was isolated.

Public health officials aimed to enhance surveillance in order to detect as many potential cases associated with the outbreak as possible. *Campylobacter* was detected in 32 stools submitted during the following 2 weeks. A cohort study (see the supplemental material) was carried out immediately, administered in person (mainly by telephone) and modified from a previously successful version under advice from Health Protection Scotland. Questions covered demographic and exposure indicators, including occupation and place of work. Self-assessed answers were used as a proxy for occupational animal exposure. A fictional exposure (to haddock) was placed in the questionnaire menu as a test of response reliability.

Environmental investigation. Environmental health officers interviewed the proprietor and employees of the event caterer, examined the premises, and traced the food chain where appropriate.

Microbiology. Presumptive *Campylobacter* isolates were obtained from hospital diagnostic laboratories on charcoal transport swabs. On receipt, each was plated onto blood agar and charcoal cefoperazone deoxycholate agar (CCDA) (CM0739; Oxoid, United Kingdom) and incubated under microaerophilic conditions (2% H_2, 5% CO_2, 5% O_2, 88% N_2) at 37°C for 48 h. Isolates were presumptively confirmed as *Campylobacter* by agglutination (Microgen, Camberley, United Kingdom) and were identified to the species level by multiplex PCR (28). One isolate failed to grow upon subculture, and thus 32 strains were forwarded for typing but 33 patients were considered for attack rate estimations.

Campylobacter isolates were serotyped (43) using heat-stable antigens prepared from fresh isolates in 3 ml sterile phosphate-buffered saline. Cell suspensions were boiled for 30 min and centrifuged at 1,500 × *g*, and supernatants were stored at 4°C. Sheep red blood cells were sensitized with 3 ml 1% sheep erythrocytes in each antigen sample. Isolate antigen-erythrocyte conjugates were screened with 50 different antisera at a working dilution (1/20).

Phage typing of *Campylobacter* isolates was performed by the overlay method (21) and recorded the degree of lysis for each isolate and assigned to the defined phage type (21).

Antibiotic susceptibility testing (53) was carried out against ampicillin (8 and 32 mg/liter), tetracycline (8 and 128 mg/liter), ciprofloxacin (1 mg/liter), nalidixic acid (16 mg/liter), erythromycin (4 mg/liter), chloramphenicol (8 mg/liter), kanamycin (16 mg/liter), neomycin (8 mg/liter), and gentamicin (4 mg/liter). When more than one antibiotic concentration was used, resistance was coded according to which concentration proved inhibitory. Isolates showing no resistance to the antibiotics tested were coded as fully sensitive.

Pulsed-field gel electrophoresis (PFGE) profiling was carried out as previously described (22). Chromosomal DNA contained in low-melting-point agarose (Sigma, Poole, United Kingdom) plugs was digested with SmaI (Invitrogen, Paisley, United Kingdom) for 6 h at 25°C. DNA restriction fragments were resolved in 1% (wt/vol) agarose gels in 0.5× Tris-borate-EDTA buffer (Invitrogen, Paisley, United Kingdom) run on a Bio-Rad CHEF-DRII system. Electrophoresis conditions were 6 V cm^{-1} for 22 h, with pulse times ramped from 10 to 35 s. Results were interpreted in accordance with accepted guidelines (51).

Multilocus sequence typing (MLST) was carried out by the method of Dingle et al. (13), with additional primers described by Miller et al. (38). Sequences were assembled using STARS software (http://pubmlst.org), and newly identified alleles and sequence types (STs) were submitted to the *Campylobacter* MLST database at the same website. Five colonies were selected for typing by MLST only for those isolates showing distinct colony morphological differences on CCDA; otherwise, one colony was selected for MLST.

The *flaA* short variable region (SVR) was sequenced as the seven MLST housekeeping loci and reaction products were purified, separated, and detected on an ABI Prism 377 automated DNA sequencer (Applied Biosystems, Warrington, United Kingdom). Sequences were assembled from the chromatograms, using the STADEN software package (48), and *flaA* SVR types were assigned using the *Campylobacter flaA* MLST database (http://hercules.medawar.ox.ac.uk/flaA/).

RESULTS

Outbreak epidemiology. Of the 165 dinner dance attendees, 164 were interviewed, 86 had symptoms of gastroenteritis, and 75 had diarrhea. Of attendees submitting stool specimens, 32 yielded a *Campylobacter* isolate. Analysis of the relative risk of developing symptoms (possible case) or diarrhea (probable case) or presenting with a confirmed *Campylobacter* infection after consuming each of 33 food items on the dinner menu showed that chicken liver paté had the highest relative risk for all three outcomes (see the supplemental material). All but one possible case and all confirmed cases shared paté consumption as a common exposure. Of the main course buffet items, chicken was the only one with a significant association with clinical illness. The consumption of soup, chives, and white bread was statistically protective: all were associated with relative risk values of <1. Two attendees reported consuming the dummy food item (haddock).

Of the 102 attendees who ate paté, 33 were animal workers (22 farmers, 4 farm workers, 6 veterinary surgeons, and 1 veterinary nurse [1 farmer was also a veterinary surgeon]) (Table 1). All farmers reported some animal exposure (comprising at least poultry).

Environmental investigation. Environmental investigation of the catering procedures established that the recipe for paté preparation involved deliberate undercooking of chicken livers by flash-frying. The paté was made in several batches, each of which was mechanically homogenized and then stored and

TABLE 2. Phenotypic and genetic typing of *Campylobacter* isolates associated with Montrose farmers' dinner dance outbreak[a]

Isolate (UoA no.)	ST	CC	*flaA* allele	HS	Resistance type (antibiotic inhibitory concn [mg/liter])	Phage type	PFGE pattern	Patient contact with animals
1945	574	574	8	15	Amp32 Tet128	—	B	−
1950	574	574	8	15	Amp32 Tet128	—	B	−
1979	574	574	8	15	Amp32 Tet8	—	B	−
1984	574	574	8	15	Amp32 Tet128	—	B	+
1988	574	574	8	15	Amp32 Tet128	—	B	−
1989	574	574	8	15	Amp32 Tet128	1	B	−
1991	574	574	8	15	Amp32 Tet128	1	B	−
1943	574	574	8	15	Amp32 Tet128	1	B	−
1948	574	574	8	15	Amp32 Tet128	1	B	+
1977	574	574	8	15	Amp32 Tet128	1	B	−
1985	574	574	8	15	Amp32 Tet128	1	B	−
2031	574	574	8	15	Amp32 Tet128	—	B	+
2034	574	574	8	15	Amp32	—	B	−
1886	574	574	8	15	Amp32 Tet8	RDNC	B	−
1926[a]	51	443	21	37	Amp32 Tet128	RDNC	C	−
1927[a]	51	443	8	37	Tet128	RDNC	C	−
1928	51	443	21	37	Amp32 Tet128	RDNC	C	−
1946	51	443	8	37	Tet128	RDNC	C	+
1975	51	443	8	37	Amp8 Tet128	RDNC	C	−
1976	51	443	8	37	Amp32 Tet128	44	C	−
1981	51	443	21	37	Amp32 Tet128	RDNC	C	−
1986	51	443	21	37	Amp32 Tet128	RDNC	C	−
2021	51	443	8	37	Tet128	RDNC	C	+
1885	257	257	16	11	Amp32 Cp Nx	2	A	−
1912	257	257	16	11	Amp32 Cp Nx	2	A	−
1914[b]	257	257	16	11	Amp32 Cp Nx	2	A	−
2058	257	257	16	18	FS	33	E	−
1925	262	21	37	1	Amp32 Tet128	—	B	+
1947	262	21	37	1	Amp32	31	E	−
1949	262	21	37	1	Amp32	31	E	−
1987	262	21	37	1	Amp32	31	E	+
1990	262	21	37	1	Amp32	31	E	−

[a] HS, heat-stable Penner serotype; FS, fully sensitive; —, no phage lysis; RDNC, reacts but does not conform; Amp, ampicillin; Tet, tetracycline; Cp, ciprofloxacin; Nx, nalidixic acid.
[b] Evidence of multiple strains (see Table 3).

served separately. The caterer's daughter subsequently presented with a confirmed case of campylobacteriosis (strain analysis data not presented here) after consuming food not served at the event. The method used for preparing the paté involved a combination of undercooking and homogenization, increasing the chance that viable *Campylobacter* organisms might have survived cooking and become dispersed throughout each batch.

Microbiology. Thirty-two *Campylobacter* isolates were identified as *C. jejuni* by multiplex PCR (29). All were examined for Penner serotype, antibiotic susceptibility, phage type, PFGE type, ST, and *flaA* SVR allele (Table 2). Good overall agreement between the six typing methods was seen (Table 2), with four clades defined by MLST, each belonging to a different clonal complex (CC). Penner and PFGE typing showed complete agreement with MLST, apart from isolate UoA-2058, where Penner typing showed a unique HS type and PFGE data aligned it with clade ST-262. SVR *flaA* results agreed with MLST for three clades but differed for five of the nine strains of the ST-51 clade, having the same value as strains in the ST-574 clade. There was poorer agreement in comparing these four typing methods against phage typing and antibiotic susceptibility results.

Two isolates (UoA-1926 and UoA-1927) yielded colonies with different morphologies on CCDA. One isolate (UoA-1914, showing no morphological differences) revealed mixed sequences on MLST and was replated on CCDA, and two colonies were then typed by MLST alone. Table 3 shows that these three isolates contained two STs each, as follows. UoA-1914 was typed as ST-257 (as originally identified) and ST-1301 (novel to the study). UoA-1926 and UoA-1927 were both typed

TABLE 3. MLST results for isolates showing coinfection

Isolate (UoA no.)	ST	CC
1914 A	257	257
1914 B	1301	692
1926 A	51	443
1926 B	51	443
1926 C	51	443
1926 D	262	21
1926 E	51	443
1927 A	262	21
1927 B	51	443
1927 C	51	443
1927 D	51	443
1927 E	51	443

as ST-51 and ST-262, which were seen previously in this study (Table 2).

There was no statistical correlation between previous exposure to animals and the phenotype or genotype of the isolates (Table 2). Representatives of each of the four MLST clades (Table 2) were seen in the 8 patients with previous occupational exposure to animals and in the 25 patients without exposure.

DISCUSSION

The epidemiological analysis strongly suggested that the chicken liver paté starter was the vehicle in this outbreak. The high risk statistically attributed to the consumption of oatcakes and marmalade was likely, on the grounds of biological plausibility, to be due to the fact that both were served with the paté. Conversely, the statistically protective effect of consumption of soup, chives, and bread and butter is presumed to be an artifact of the first course being a mutually exclusive choice (of either the paté course or the soup course) rather than any biologically protective effect. Unequivocal evidence of source attribution was hindered due to the caterer's family having consumed all remaining paté, leaving none for microbiological testing.

Strain typing by MLST has been used to investigate the diversity in *Campylobacter* strains within hosts, host-associated alleles, spatial epidemiology, and population structures (18, 35, 37). This outbreak revealed four STs (Table 2), with each (not including ST-1301) having known associations with both human gastroenteritis and retail chicken, as reported in the public *Campylobacter* MLST database (http://pubmlst.org) and/or the Aberdeen University *Campylobacter* MLST database, containing approximately 6,000 Scottish clinical and environmental isolates (17). The Aberdeen University database also showed that three of the STs (ST-257, ST-51, and ST-574) were the first, fifth, and sixth most common clinical strains, respectively, during the outbreak reporting period. The fourth ST (ST-262) was associated with cattle, but CC-21, to which ST-262 belongs, has a diverse association, which includes ruminants and retail chicken. ST-1301 has been reported only once previously, from a wild avian source, and therefore its pathogenicity to humans is currently unknown.

Sufficient chicken liver paté to cater for 165 people (the number of attendees at this function) required livers from many individual birds. The prevalence of *Campylobacter* in UK retail chicken is high (24), and unpublished data from our laboratory showed chicken livers to be equally contaminated (17). Birds at broiler farms can harbor multiple *Campylobacter* strains (7), and it is well known that further cross-contamination can occur during processing (2, 41). In this outbreak, there was no evidence that the birds from which the livers were derived were from the same flock, so it is reasonable to assume that the range of isolates from the livers was as diverse as that in retail birds purchased at random. Additional contamination from potentially different *Campylobacter* strains could also have occurred at the butcher (prior to purchase) and during paté preparation in the kitchen. The paté was reportedly made in several batches and stored separately, so it is likely that each batch (if each, as reported, was prepared improperly by undercooking) contained a subset of *Campylobacter* strains. We concluded that attendees who ate paté (from one or more batches) most probably ingested multiple *Campylobacter* strains.

Stools submitted for microbiological investigation are screened routinely for *Campylobacter* by being streaked onto selective agar prior to visual assessment of target colonies for phenotypic confirmation. In this study, one isolate colony was typed unless morphological differences among colonies from the same isolate were observed or mixed traces occurred in MLST. We have no indication that a single typed strain was the one responsible for infection. Bacterial infections are usually attributed to a single strain of one specific pathogen, although exceptions have been reported. Coinfection of *C. jejuni* and *E. coli* O157 was associated with a waterborne outbreak (6), and Richardson et al. (44) found that four (7.5%) persons with sporadic cases of campylobacteriosis were coinfected with two strains of *C. jejuni*, as confirmed by molecular typing. Our study revealed three patients with coinfections. Whether isolates not showing morphological differences among colonies also comprised mixed strains was not investigated, and therefore the degree of coinfection may be underreported here. Due to the findings here, together with those of Richardson et al. (44), consideration should be given to multiple strain typing of *Campylobacter* to confirm outbreak sources and cases of coinfection. Data from highly discriminatory typing such as MLST may contradict the outbreak strain view of point source outbreaks, particularly from chicken products, where multiple strain carriage is recognized.

Outbreak investigations and case-control studies on *Campylobacter* have identified consumption and handling of raw chicken as key risk factors for human illness (3, 19). Paté dishes from meat have been associated with a range of microbial pathogens, including *Salmonella* (52), *Listeria* spp. (11), and *E. coli* O48 (14). Gillespie et al. (23) suggested that infection from *C. coli* was more likely to be linked to paté consumption than was *C. jejuni* infection. Although they could find no scientific reports on *Campylobacter* outbreaks directly attributed to chicken liver paté itself, Layton et al. (33) described a mixed *C. jejuni* and *Salmonella enterica* serovar Heidelberg outbreak where a food-handling error connected to cooked chicken livers occurred. Furthermore, two additional *Campylobacter* outbreaks linked to paté consumption recently occurred in Scotland (25, 26), although as in this case, no strains were isolated from remaining food.

This outbreak highlights the danger of *Campylobacter* infection from eating undercooked chicken products. Although costly in time and consumables, the typing of multiple colonies from a single source identified coinfections here, as determined by MLST. We found that for the basis of a short-term epidemiological investigation, the combined use of genotypic and phenotypic subtyping techniques allowed efficient, reproducible grouping of isolates. The phenotypic methods gave results which correlated well with those of the typically more discriminatory sequence-based techniques. In order to fully understand the complex epidemiology of *Campylobacter*, it is important to capitalize on outbreak events such as these, which help to identify infection sources and ultimately to reduce morbidity.

This study provides some evidence of acquired immunity to campylobacteriosis. Patients may have ingested multiple strains but presented with one to which they had no prior

exposure. This is speculative due to patient serological information being outside the scope of this investigation. However, many of the people attending the dance were from farming backgrounds, and the epidemiological investigations showed that these groups were less likely to show illness than those not in regular contact with animals (Table 1). The results of opportunistic subgroup analysis in the context of the large and unusual outbreak presented here are congruent with and reinforce the previously suggested increased immunity to further infection from *Campylobacter* conferred by exposure to animals. Assuming that such acquired immunity is a genuine effect, it raises the possibility of cross-immunity since it seems unlikely, given the known diversity in environmental and potentially zoonotic *Campylobacter* strains (12), that previous exposure will consistently have involved outbreak strains. There is experimental evidence of such cross protection (8). If this does indeed occur, then perhaps the development of an effective *Campylobacter* vaccine (30, 54) is realistically achievable.

This outbreak highlights the public health issues from paté preparation by deliberate undercooking of chicken livers, which in this case led to an unusually large outbreak of campylobacteriosis. We suggest that large volumes of paté made from many contaminated chicken livers were responsible for coinfections with multiple *Campylobacter* strains. These data also suggest that occupational animal exposure confers statistically significant protection against diarrheal illness after food-borne exposure to *C. jejuni*.

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