Biomarkers, June 2008; 13(4): 413–421

informa
healthcare

Identification of *Campylobacter* infection in chickens from volatile faecal emissions

CATHERINE E. GARNER[1,5], STEPHEN SMITH[1], NICOLA C. ELVISS[2,3], TOM J. HUMPHREY[4], PAUL WHITE[1], NORMAN M. RATCLIFFE[1], & CHRISTOPHER S. PROBERT[5]

[1]*Centre for Research in Analytical, Materials, and Sensor Sciences, University of the West of England, Coldharbour Lane, Bristol BS16 1QY, UK,* [2]*Food Borne Zoonoses Unit, Health Protection Agency, School of Clinical Veterinary Science, University of Bristol, Langford BS40 5DU, UK,* [3]*Food, Water and Environmental Microbiology Laboratory, Health Protection Agency Yorkshire & the Humber, Bridle Path, York Road, Leeds LS15 7TR, UK,* [4]*Zoonotic Infections Group, School of Clinical Veterinary Science, University of Bristol, Langford BS40 5DU, UK and* [5]*Clinical Science at South Bristol, Bristol Royal Infirmary, Marlborough St, Bristol, BS2 8HW, UK*

Abstract

Volatile organic compounds from chicken faeces were investigated as biomarkers for *Campylobacter* infection. *Campylobacter* are major poultry-borne zoonotic pathogens, colonizing the avian intestinal tract. Chicken faeces are the principal source of contamination of carcasses. Fresh faeces were collected on farm sites, and *Campylobacter* status established microbiologically. Volatile organic compounds were pre-concentrated from the headspace above 71 separate faecal samples using solid-phase microextraction and separated and identified by gas chromatography/mass spectrometry. A *Campylobacter*-specific profile was identified using six of the extracted volatile organic compounds. The model developed reliably identified the presence or absence of *Campylobacter* in >95% of chickens. The volatile biomarker identification approach for assessing avian infection is a novel approach to enhancing biosecurity in the poultry industry and should reduce the risk of disease transmission to humans.

Keywords: Campylobacter, *chickens, volatile organic compounds, gas chromatography, solid-phase microextraction, biosecurity*

(Received 18 September 2007; accepted 4 February 2008)

Introduction

Infection with *Campylobacter* spp. is reported in approximately 50,000 people annually in England and Wales (Health Protection Agency 2006), although up to 1% of the population is probably affected each year (Wheeler et al. 1999). In many cases, the source of infection is poultry meat (Friedman et al. 2004). Campylobacteriosis is often

Correspondence: Norman M. Ratcliffe, Centre for Research in Analytical, Materials, and Sensor Sciences, University of the West of England, Coldharbour Lane, Bristol BS16 1QY, UK. E-mail: Norman.Ratcliffe@uwe.ac.uk

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500801966443

mild, but serious, even life-threatening complications may occur. *Campylobacter jejuni* is the most common pre-disposing factor for Guillain–Barré syndrome (Rees et al. 1995), a paralysing disease affecting the nerves to the arms, legs, head and trunk. In addition, there are gastrointestinal complications including toxic megacolon with an attendant risk of perforation or colectomy (Schneider et al. 2000) as well as postinfectious irritable bowel syndrome (Parry et al. 2003).

Control of *Campylobacter* in chickens on-farm is central to reducing the burden of human disease and biosecurity is the principal means of control. Rapid detection of *Campylobacter* in chicken flocks would aid control and may enable segregation of positive and negative flocks prior to slaughter, as has been recommended (Berndtson et al. 1996).

Volatile organic compounds (VOCs) emitted from faeces can be readily identified and used to determine the presence of specific bacteria in human stools (Garner et al. 2007). If chicken faeces' VOCs have a specific pattern, the approach may provide a rapid means of screening chickens for *Campylobacter* compared with current microbiological methods that take several days to produce a definitive result. There is little literature relating to VOCs emitted from chicken faeces. We undertook a pilot study to identify a wide range of the volatile emissions from chicken faeces with and without *Campylobacter*.

Materials and methods

Freshly voided faecal samples were collected from chickens from three poultry farms associated with the same production company in Southwest England between October 2004 and February 2005.

The farms were designated A, B and C and 31, 20 and 20 samples were collected, respectively. The chickens were the same breed, fed the same grain-based diet from the same supplier, and reared on a litter of wood shavings. The areas outside, to which the chickens had access, were grassed. All samples were returned to the laboratory within 2 h of collection.

Fresh faecal samples were collected from the floor of the free-range housing and placed directly into sterile 20 ml plastic containers and returned to the laboratory. Faeces were located from the chickens when freshly deposited due to the large numbers contained in the housing.

At the laboratory, the containers were opened under aseptic conditions and $\sim$2 g of faeces transferred to a 25 ml Supelco vial sealed with a phenolic screw top and PTFE/silicone septa and immediately frozen. The remaining sample was examined for the presence of *Campylobacter*.

Detection of Campylobacter

A portion of each faecal sample was cultured on selective modified charcoal cefoperazone deoxycholate agar (Oxoid, Basingstoke, UK) for *Campylobacter* identification. All samples were incubated in a microaerobic atmosphere (10% CO_2, 10% H_2 and 80% N_2) at 37°C for 48 h (Humphrey et al. 2005).

The isolation protocols for *Campylobacter* in chicken faecal samples are internationally recognized and highly sensitive. Each batch of faecal samples included both

positive and negative controls. In addition, *Campylobacter* achieve high numbers in infected chickens and there is only a very small chance of false negatives.

Instrumentation

Two gas chromatography/mass spectroscopy (GC/MS) systems were used: Hewlett Packard GC (model 6890), with a 0.75 mm quartz liner linked to a bench-top quadrupole MS (Model 5973), and a Perkin Elmer Clarus 500 GC/MS quadrupole bench-top system, with a 1 mm quartz liner. Importantly, the same column was used throughout the study, which comprised two conjoined columns: a SPB-1 sulphur fused-silica capillary column, 30 m $\times$0.32 mm ID $\times$4.0 μm film thickness, (Supelco, Poole, UK) and a ZB-FFAP fused-silica capillary column, 30 m $\times$0.32 mm ID $\times$0.50 μm film thickness (Phenomenex, Macclesfield, UK). Helium carrier gas (99.9995%; BOC, Guilford, UK) was run through an Excelasorb™ helium purification system (Supelco) at 1.16 ml/min. The utilized method, using two GC/MS systems, had already been tested on human faecal samples in disease diagnosis (Garner et al. 2007). The SPME fibres were assessed by running a blank on a daily basis.

Glass vials containing the chicken faeces were removed from the freezer at -20°C and placed in a water bath at 60°C for 1 h. Carboxen/polydimethylsiloxane (CAR/PDMS) SPME fibres were used for VOC extraction (Probert et al. 2004) and were inserted into the vial headspace above the faeces and exposed to the volatiles, without stirring or agitation, for 20 min. After removal, the fibre assembly was transferred immediately to the GC injection port for desorption at 280°C with the split valve closed throughout. The GCs were operated under the following conditions: solvent delay 4 min; temperature programme 35°C (5 min), ramp of 7°C min^{-1} to 250°C and then held at 250°C for 12 min, giving a total run time of 47.71 min. The MS was operated in EI mode scanning from mass charge ratio 17–350 (4–47.71 min). VOCs were identified using Amdis software to separate convoluting peaks, matching the retention times, standard comparison where appropriate and using the NIST (05) library. The concentration of each VOC was not evaluated as the SPME carboxen fibres have been shown to extract in differing concentrations depending upon the availabilities of the VOCs in the mixture (Cho et al. 2003). However it is reasonable to compare VOC profiles from different samples where the sampling and analytical protocols are the same.

The presence or absence of each VOC was recorded and coded '1' or '0', respectively, for each sample. Two-way cross-tabulations between farms (A, B and C) and volatile status (present, absent) were performed for each volatile to identify farm-specific ones. For each non-farm-specific volatile, the volatile status (present, absent) was cross-tabulated against that of *Campylobacter* (negative, positive) and Pearson's χ^2 test of association was performed to identify any significant associations with the presence of the *Campylobacter* in faeces. Standard multivariate discriminant analysis is a well-established classification technique and was used to identify those volatiles whose presence or absence best multivariately discriminates between positive cases and negative cases (see for instance Chapter 11 of Tabachnick & Fidell 2001). Forward stepwise discriminant analysis was used to identify a parsimonious subset of non-farm-specific volatiles that multivariately discriminate between positive and negative faeces. The dependent variable in the discriminant analysis was *Campylobacter* status (positive, negative) and the potential set of independent variables in the

analysis were the non-farm-specific volatile compounds dummy variable coded '1' for present and '0' for absent. Model validation was performed using the leave-one-out (LOO) technique.

Results

Number of VOCs identified

One hundred and twenty-five different volatile organic compounds were identified in the headspace above all of the faecal samples using a criterion of greater than 79% match. It was not possible to include all the peaks where the spectral pattern of the compounds in question did not match at the same retention time, and therefore were not included in this analysis. The microbiological assessment showed that in farm A, none of the 31 samples collected were contaminated with *Campylobacter*, from farm B, all 20 samples contained *Campylobacter* and from farm C, 20 samples were obtained, 10 with, and 10 without *Campylobacter.* The levels of *Campylobacter* spp. in the chickens was high; $>10^6$ g^{-1} of faeces. The type of *Campylobacter* was usually found to be *C. jejuni*. The mean number of compounds emitted was similar for samples from each farm (43, 31 and 33, for farms A, B and C, respectively). Similarly, the mean number of compounds was similar from samples with and without *Campylobacter* (33 and 37, respectively). Figures 1 and 2 provide sample chromatograms for *Campylobacter*-infected faeces and healthy controls, respectively. Volatile compounds found in chicken faeces from *Campylobacter*-negative and *Campylobacter*-positive chickens, showing statistically significant differences with $p \leq 0.05$ are listed in Table I.

Profile of VOCs on each farm

The VOC profile was compared for each farm as well as for chickens with or without *Campylobacter*. It was considered probable that the conditions on each farm, such as feed and housing, may influence the VOC profile. This was confirmed when the VOC

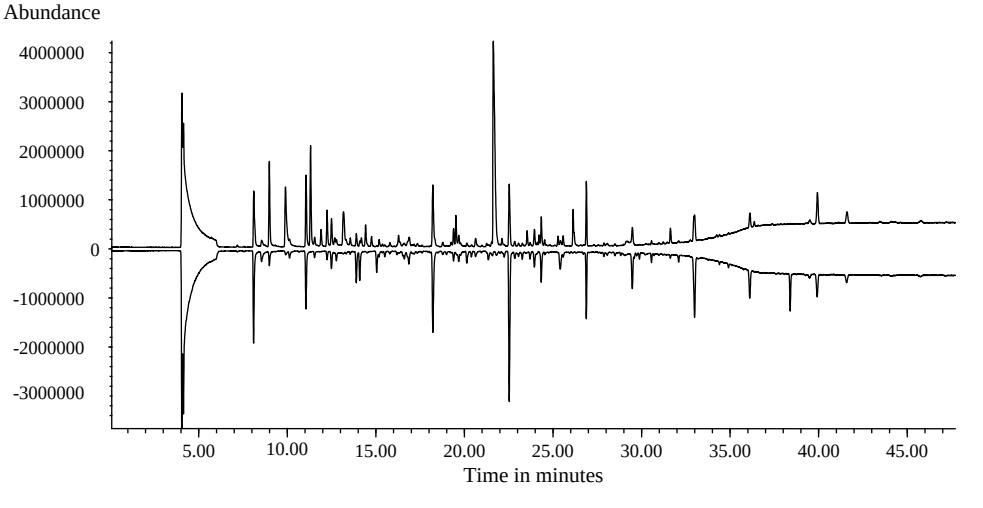

Figure 1. Comparison of two chromatograms from the headspace VOCs of faeces from *Campylobacter*-infected chickens.

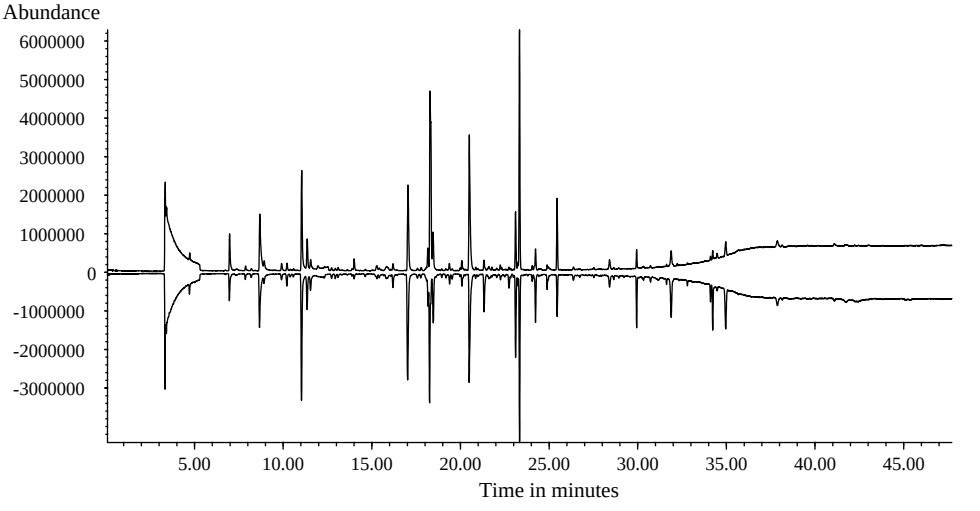

Figure 2. Comparison of two chromatograms from the headspace VOCs of faeces from healthy controls in chickens.

profile of each farm was studied. The presence of several key compounds was shown to be strongly associated with each farm. Limonene and 4-methylphenol were present in the faeces from all chickens from farm B, from which all samples were *Campylobacter*-positive, and were not found in any sample from other farms. Several compounds, e.g. 2-methyl-2-pentanone and phenylethyl alcohol, were absent from the profile of samples from farm C, while being present in most of the samples from farms A and B. Such markers of specific farms may have a role in linking chickens to specific farms and might be useful should chickens have been moved between farms prior to slaughter.

Profile of VOCs with or without Campylobacter

Given the strong relationship between farm B (in which all chickens were *Campylobacter*-positive) and limonene and 4-methylphenol, we were concerned that this relationship was absent from samples from farm C (in which 50% of chickens had *Campylobacter*), which suggested that these compounds may have been specific to *Campylobacter* but that they were either not sensitive enough to use, or alternatively that they were farm specific. In order to identify compounds that were specific to this bacteria and not arising because of the farm of origin, the compounds that were potential markers of a farm were eliminated from the analysis. Forty-nine such compounds were removed. Five non-farm-specific compounds were found that were significantly ($p<0.05$) more common in *Campylobacter*-positive samples than in negative ones; 2-hexanone, (E)-3-hexen-2-one, hexanal, (E)-2-octenal and pyrrole. There were 24 compounds that were significantly less common in the positive than the negative samples; at the $p<0.01$ level for 15 of these (see Table I). None of the compounds, in isolation, were uniquely associated with the presence or absence of *Campylobacter* so discriminant analysis was undertaken.

Forward stepwise discriminant analysis identified six VOCs: hexanal, (E)-2-octenal, pyrrole, ethyl ethanoate, methyl alcohol and 2-heptanone that each significantly

Table I. Marker volatile compounds found in chicken faeces from *Campylobacter*-negative and -positive chickens.

Volatile	% of Positives	% of Negatives	*p*-Value (using χ^2)
2-Hexanone	37	2	<0.001
3-Hexen-2-one	53	12	<0.001
2-Butenal	7	61	<0.001
Methyl butanoate	3	66	<0.001
Ethyl ethanoate	50	93	<0.001
Hexanoic acid	17	68	<0.001
Methyl alcohol	10	83	<0.001
4-Ethylphenol	7	46	<0.001
Propanal	20	66	<0.001
Propanoic acid	27	73	<0.001
3-Methyl-2-butanone	3	37	0.001
Butyrolactone	3	37	0.001
n-Propyl ethanoate	10	44	0.002
3-Hydroxy-2-butanone	63	93	0.002
Methyl pentanoate	0	24	0.004
Methyl propionate	37	71	0.004
1-Propanol	30	63	0.005
1-Penten-3-one	40	71	0.010
Ethyl propanoate	13	42	0.010
Phenol	43	73	0.011
1-Butanol	17	44	0.015
Hexanal	100	83	0.017
(E)-2-Octenal	23	5	0.021
1-Penten-3-ol	10	32	0.031
3-Octanol	63	85	0.032
1-Octen-3-ol	83	98	0.033
2,3-Butanedione	90	100	0.039
2-Heptanone	80	95	0.047
Pyrrole	77	54	0.047
2,5-Dimethylpyrazine	47	24	0.050
2,3-Pentanedione	77	93	0.055
3-Pentanone	80	59	0.056

contribute to the multivariate discrimination between positive and negative cases. The derived linear classification function is estimated to be:

$$Z = 4.7 + 3.937 \times \text{Hexanal} + 5.825 \times [\text{(E)-2-Octenal}] + 5.375 \times \text{Pyrrole} \\ -5.413 \times \text{Ethyl ethanoate} - 7.958 \times \text{Methyl Alcohol} - 8.033 \times [\text{2-Heptanone}]$$

In the above equation the VOC name takes the value '1' if the VOC is present in a sample and '0' otherwise. The above equation can be evaluated for any given present/absent profile and an overall positive score ($Z > 0$) is indicative of a positive case whereas an overall negative score is indicative of a negative case.

Accuracy of the model

When the model was tested against the result for chickens with *Campylobacter*, it was correct in 29/30 cases. When tested against chickens without the infection, the model was accurate in 39/41 cases. In other words, the accuracy was highly significant ($\chi^2 =$

59.3, df = 1, $p < 0.000001$); it had a sensitivity of 96.7% and specificity of 95.1%, the positive predictive value was 93.5% and the negative predictive value was 97.5%. Figure 3 is a comparative box-and-whiskers plot summarizing the classification scores for the sample positive and negative cases.

An indication of the out-of-sample predictive accuracy can be obtained by conducting a LOO cross-validation. In LOO a case is temporarily omitted from the dataset, the remaining data are used to re-fit the model and the new model used to predict the case temporarily omitted. For the sample data a LOO cross-validation analysis indicates that this model is a robust predictor with only two additional cases (both negative) being misclassified and in all instances the volatiles in the classification function remain statistically significant

Discussion

Conventional culture-based methods for detecting *Campylobacter* require up to 4 days to confirm the presence of the bacterium. The method requires specialized media, the ability to generate the microaerophilic atmosphere required for the bacterium to grow and involves a high labour input from trained laboratory staff. Although molecular-genomic methods such as polymerase chain reaction (PCR) can reduce the detection time to 8 h, standard PCR provides no information about the viability of the cells (Stelzer et al. 1989). The ability to use the volatile profile means that the samples can

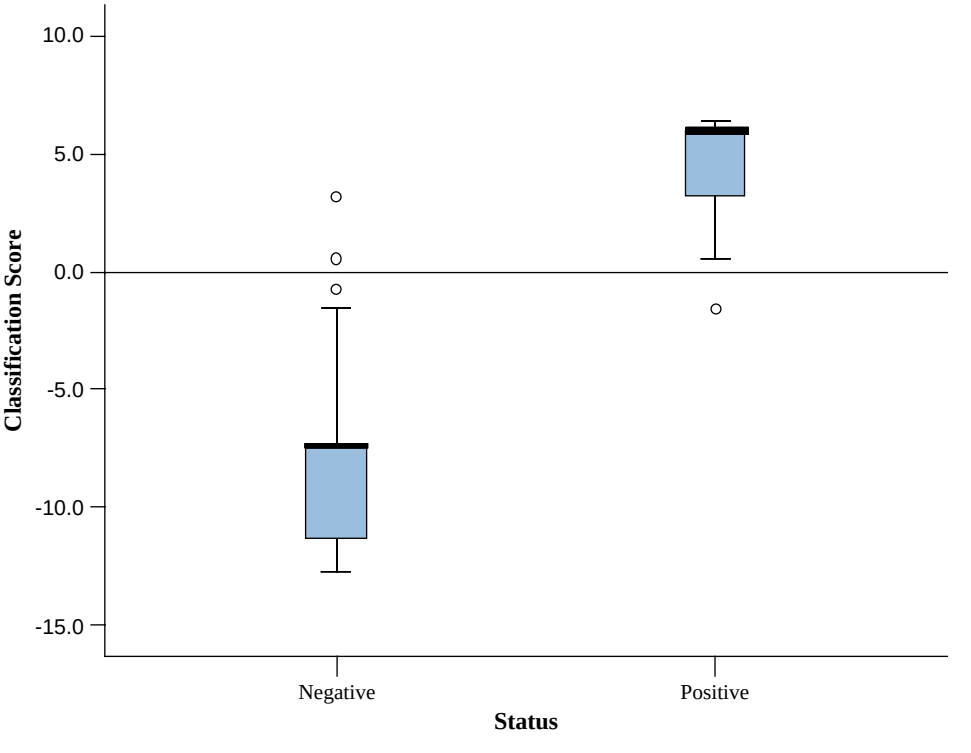

Figure 3. A comparative box-and-whiskers plot summarizing the classification scores for the cases of *Campylobacter*-positive and -negative samples in chickens.

be collected and frozen immediately allowing them to be transported in a controlled environment to the laboratory. Sampling for the volatiles is more rapid than conventional culture, taking just 2 h to process a sample. Our method was not optimized and it is quite likely that the 60 min heating time and the chromatography time could be significantly reduced. A costly GC/MS system was used for identification of metabolite biomarkers, once these are established a simpler system with sensors targeted to key volatiles can be envisaged (Cowell & Ratcliffe 1997), for use on-site, or alternatively a much lower cost laboratory-based GC fitted with a sensitive detector with a broad selectivity, such as a flame ionization detector.

In this study we have identified 125 different volatile organic compounds, in total, emitted from the faeces of chickens. Figures 1 and 2 show visually the similarity between the chromatograms of two different samples with *Campylobacter* and two different samples of healthy control chicken faeces. It should also be noted that the chromatograms appear very different in the two sets. The profiles of the VOCs were compared in two ways: (1) to compare chickens with and without *Campylobacter* and (2) to compare farms.

Each flock had a subtly different VOC profile; indeed the difference could be used to distinguish one farm from another. Such differences were anticipated and likely arise from the environment in which the chickens are reared and its impact of the environment on the intestinal flora of the chickens. Each flock was fed commercial feed as well as being allowed to range on open grass. Cleaning agents and other compounds used on the farms may also contribute to the profile associated with each farm. When analysing the data, all of the farm A were correctly predicted and all of the farm B were correctly predicted using the derived formula. The three incorrect predictions were all farm C, two negatives are predicted to be a positive; and one positive predicted to be a negative.

The samples identified had either *Campylobacter jejuni* or *C. coli*, both of which are capable of producing food poisoning in man, we have not differentiated between species in order to provide a VOC signature which would be widely applicable for assessing disease-carrying chickens.

We were able to show clear differences in the VOC profile between chickens with *Campylobacter* and those without the infection. Table I provides strong support that the VOC faecal profiles between those with and without *Campylobacter* are appreciably different. Six compounds were identified which, when used together, could be used to classify faecal samples as positive or negative for *Campylobacter*. Some of these compounds were associated with the infection and some were present in chickens without *Campylobacter*. The model was highly statistically significant: the sensitivity and specificity were high and this model is likely to be useful to industry and regulatory authorities. Further studies to quantify VOC concentrations would be of value to provide greater discrimination.

These data indicate that the hypothesis, that the VOC profile will be characteristically different in chickens with *Campylobacter*, was correct. VOC profile could be used to distinguish those chickens that have *Campylobacter* from those that do not. We propose a new approach to the detection of *Campylobacter* using sensors sensitive to the change in VOCs; we believe that sensors could be built to respond to these compounds and could be used by industry to support biosecurity and reduce contamination of chicken meat.

Acknowledgements

We would like to thank the Engineering and Physical Sciences Research Council for funding a PhD studentship for Katy Garner.

References

Berndtson E, Emanuelson U, Engvall A, Danielsson-Tham M-L. 1996. A 1-year epidemiological study of *Campylobacter* in 18 Swedish chicken farms. Preventive Veterinary Medicine 26:167–185.

Cowell DC, Ratcliffe N. 1997. The detection of micro-organisms in foodstuffs by the use of vapour sensors. Measurements and Control – UK 30:39–42.

Cho H-J, Baek K, Lee H-H, Lee S-H, Yang J-W. 2003. Competitive extraction of multi-component contaminants in water by carboxen–polydimethylsiloxane fiber during solid-phase microextraction. Journal of Chromatography A 28:177–184.

Friedman CR, Hoekstra RM, Samuel M, Marcus R, Bender J, Shiferaw B, Reddy S, Ahuja SD, Helfrick DL, Hardnett F, Carter M, Anderson B, Tauxe RV. 2004. Risk factors for sporadic *Campylobacter* infection in the United States: A case-control study in FoodNet sites. Clinical Infectious Diseases 38 (Suppl. 3):S285–S296.

Garner CE, Smith S, de Lacy Costello B, White P, Spencer R, Probert CS, Ratcliffe NM. 2007. Volatile organic compounds from feces and their potential for gastrointestinal disease diagnoses. FASEB J 21:1675–1688.

Health Protection Agency. *Campylobacter* spp. Laboratory reports of faecal isolates England & Wales; 1986–2005. Available from: http://www.hpa.org.uk/infections/topics_az/campy/data_ew.htm [Last accessed 18 February 2008].

Humphrey TJ, Jorgensen F, Frost JA, Wadda H, Domingue G, Elviss NC, Griggs DJ, Piddock LJ. 2005. Prevalence and subtypes of ciprofloxacin-resistant *Campylobacter* spp. in commercial poultry flocks before, during, and after treatment with fluoroquinolones. Antimicrobial Agents and Chemotherapy 49:690–698.

Parry SD, Stansfield R, Jelley D, Gregory W, Phillips E, Barton JR, Welfare MR. 2003. Does bacterial gastroenteritis predispose people to functional gastrointestinal disorders? A prospective, community-based, case-control study. American Journal of Gastroenterology 98:1970–1975.

Probert CSJ, Jones PRH, Ratcliffe NM. 2004. A novel method for rapidly diagnosing the causes of diarrhoea. Gut 53:58–61.

Rees JH, Soudain SE, Gregson NA, Hughes RA. 1995. *Campylobacter jejuni* infection and Guillain-Barré syndrome. New England Journal of Medicine 333:1374–1379.

Schneider A, Runzi M, Peitgen K, von Birgelen C, Gerken G. 2000. *Campylobacter jejuni*-induced severe colitis – a rare cause of toxic megacolon. Z Gastroenterology 38:307–309.

Stelzer W, Mochmann H, Richter U, Dobberkau HJ. 1989. A study of *Campylobacter jejuni* and *Campylobacter coli* in a river system. Zentrablatt fur Hygiene und Umweltmedizin 189:20–28.

Tabachnick BG, Fidell LS. 2001. In: Using Multivariate Statistics, 4th edn. Boston, MA: Allyn and Bacon.

Wheeler JG, Sethi D, Cowden JM, Wall PG, Rodrigues LC, Tompkins DS, Hudson MJ, Roderick PJ. 1999. Study of infectious intestinal disease in England: rates in the community, presenting to general practice, and reported to national surveillance. British Medical Journal 318:1046–1050.