Epidemiol. Infect. (2011), **139**, 1361–1368. © Cambridge University Press 2010
doi:10.1017/S0950268810002359

Seroepidemiological studies indicate frequent and repeated exposure to *Campylobacter* spp. during childhood

C. W. ANG[1]*, P. F. M. TEUNIS[2,3], P. HERBRINK[4], J. KEIJSER[4], Y. H. T. P. VAN DUYNHOVEN[5], C. E. VISSER[6] AND W. VAN PELT[2]

[1] *Department of Medical Microbiology and Infection Control, VUMC Amsterdam, The Netherlands*
[2] *Epidemiology and Surveillance Unit, Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands*
[3] *Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, GA, USA*
[4] *Diagnostic Centre, SSDZ, Delft, The Netherlands*
[5] *Laboratory for Zoonoses and Environmental Microbiology, Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, The Netherlands*
[6] *Department of Medical Microbiology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands*

(*Accepted 15 September 2010; first published online 26 October 2010*)

SUMMARY

The annual number of episodes of clinical gastroenteritis caused by *Campylobacter* spp. in The Netherlands is estimated to be 75 000, i.e. once per 200 person life-years. This number is based on extrapolation of culture results from population-based studies. The number of culture-confirmed cases of *Campylobacter* infection peaks in the first 3 years of life and again between the ages of 20 and 25 years. The seroepidemiology of *Campylobacter* describes the relationship between age and exposure to *Campylobacter* and reflects both symptomatic and asymptomatic infections. Using a validated ELISA system, antibodies to *Campylobacter* were measured in an age-stratified sample ($n=456$) of the PIENTER serum collection of the Dutch general population. The seroprevalence of *Campylobacter* IgG antibodies increased with age, reaching almost 100% at age 20 years. Antibody levels steadily increased with age until young adulthood, suggesting repeated exposure to *Campylobacter*. In conclusion, seroepidemiological data demonstrated repeated exposures to *Campylobacter* throughout life, most of which do not lead to clinical symptoms. From young adulthood, >95% of the population in The Netherlands had serological evidence for exposure to *Campylobacter*.

Key words: *Campylobacter*, foodborne infection, gastrointestinal infections, mathematical modelling, serology.

INTRODUCTION

Campylobacter spp. are the most frequently identified pathogens in patients with gastroenteritis. The health burden is high and is estimated to be 1800 disability-adjusted life years (DALY) per year in The Netherlands (population 16 million) [1]. This is mainly caused by post-infectious sequelae such as reactive arthritis and Guillain–Barré syndrome [2]. In industrialized countries, the epidemiology of *Campylobacter* infections is built upon population-based culture studies [3, 4]. These studies yield a figure

* Author for correspondence: Dr C. W. Ang, Department of Medical Microbiology and Infection Control, VUMC Amsterdam, The Netherlands.
(Email: w.ang@vumc.nl)

of 75 000 symptomatic infections annually in The Netherlands, i.e. once per 200 person life-years [3]. The number of positive *Campylobacter* cultures peaks in the first years of life with a significant later peak in young adults, especially females [5, 6].

In developing countries, infection pressure is much greater and the number of infections is consequently much higher, varying from 0·4 to 1·5 symptomatic *Campylobacter* infections per person annually [7–9]. Furthermore, children in developing countries are repeatedly infected with new strains although many of these infections are asymptomatic [8]. Interestingly, in both developed and developing countries, very young children have the highest rate of symptomatic infections, which declines rapidly in the first 3 years of life. *Campylobacter* infections in the first year of life are most often associated with bloody diarrhoea, whereas at higher ages infections are generally mild.

Serological methods are of great help when investigating infectious disease epidemiology. Very few seroepidemiological studies into *Campylobacter* infections have been performed and the methodology used in these studies is not always straightforward [10, 11]. In the current study, we describe the seroprevalence of *Campylobacter* in a sex- and age-stratified sample of the general population of The Netherlands. Using a binary mixture model, we show that it is possible to estimate the infection pressure of *Campylobacter* in the Dutch population.

METHODS

Patients

Serum samples were derived from the PIENTER database [12]. Briefly, in order to establish a serum bank of the Dutch general population, eight municipalities with probabilities proportional to their population size were sampled within each of five geographical Dutch regions with similar population sizes. For the complete PIENTER database, an age-stratified sample (age groups <1, 1–4, 5–9 …, 75–79 years) of 380 individuals was randomly selected from each municipality. In each of the first two strata 40 individuals were sampled, while in each of the following strata 20 individuals were sampled. Subjects were requested to give a blood sample and to complete a questionnaire. From all individuals information on gender and urbanization was also included in the database. Samples and data were collected during the period from October 1995 to December 1996. The response rate was 55%. The original national serum bank included 8359 sera of which 456 were analysed for this study.

We randomly selected these 456 samples stratified by 21 categories with almost equal numbers for age (0, 1–4, 5–14, 15–34 males, 15–34 females, 35–64 and >64 years) and three degrees of urbanization (low, intermediate, high). With this age stratification, there are relatively more young children in our sample because this age group has the highest rate of symptomatic infections [13]. Based on seroprevalence rates for IgG from Belongia *et al*. [10] hypothetical datasets of increasing size were analysed as described in the following section. Age and degree of urbanization became significant at a sample size of 210. We also took into account the uncertainties regarding different assay systems for detection of anti-*Campylobacter* antibodies and the different nature of the population, and estimated that a sample of at least twice the calculated sample size of 210 would suffice.

Serological testing of samples

IgA, IgM and IgG antibody reactivity against *Campylobacter* spp. was measured using an ELISA as described previously [14]. The antigen used for the ELISA was an acid glycine extract of *C. jejuni* strain SSDZ-01. Data for all isotypes were expressed as ratios. The ratio was calculated by dividing the mean optical density (OD) value of the serum, tested in duplicate, by the median OD value of the reference sample that was included in triplicate on each ELISA plate. Specificity of the *Campylobacter* ELISA was investigated by pre-incubating serum samples from individuals with documented *Campylobacter* infection and healthy controls with known anti-*Campylobacter* IgG reactivity with bacterial suspensions of the following species: *C. jejuni*, *C. coli*, *C. upsaliensis*, *C. lari*, *C. fetus*, *C. hyointestinalis*, *Helicobacter pylori*, *Legionella pneumophila*. After pre-incubation the samples were centrifuged and further tested by ELISA. Results from the inhibition ELISA are expressed as OD values.

Statistical analysis and modelling

Describing the dependencies on age, we attempted to impose small *a priori* constraints on the shapes of the age dependencies of the serology measurements and *Campylobacter* incidence by modelling with spline functions [15]. Sex and degree of urbanization were

https://doi.org/10.1017/S0950268810002359 Published online by Cambridge University Press

added to the model as covariables and as interaction terms with the splines in order to study the age dependency in males and females, and in urban and rural areas. The significance of the contribution of the covariables, splines and interaction terms was tested with the multivariate Wald test [16]. Using the estimated model parameters, the overall average changes with age in the various subgroups were plotted, together with 90% confidence limits. Informally this allows evaluation of the significance of differences in level between subgroups at different ages, comparable to an α of $\sim$0·05. Calculations were performed in Dyalog APL v. 8.2.5 (Dyalog, UK).

Estimates of the seroprevalence were based on classification of sera into (sero)negatives and (sero)positives by means of a binary distribution mixture approach [17]. Observed ratios were log-transformed and a binary mixture of normal distributions was fitted by means of maximum likelihood. In short, the serological sample can be classified into two subclasses (labelled positive and negative). Either subclass can be described by a lognormal distribution of OD values. The positive subclass has higher OD values than the negative subclass (this is not a real assumption, only a definition to avoid ambiguity in identifying subclasses). Subclasses are independent of age (i.e. age groups may have different numbers of seropositives but the shapes of the positive and negative components are the same). No prior assumptions were made about the means and variances of either distribution. These characteristics were estimated from the observed data, as well as the number in either class – the seroprevalence. Note that this classification method does not assume that any individual in the observed population should have the same threshold (i.e. being classified as seropositive). Rather, any individual observed OD is interpreted as having a probability of being positive that is a function of the observed OD value. Therefore the binary distribution mixture method does not ignore heterogeneity in serological response in individual subjects. In age-stratified samples the seroprevalence (i.e. the number positive) was estimated for each age group while mixture components were kept the same for all ages. Numerical procedures were programmed in Mathematica v. 6.0 (Wolfram Research, USA).

RESULTS

To investigate the specificity of the *Campylobacter* ELISA we performed inhibition ELISA experiments.

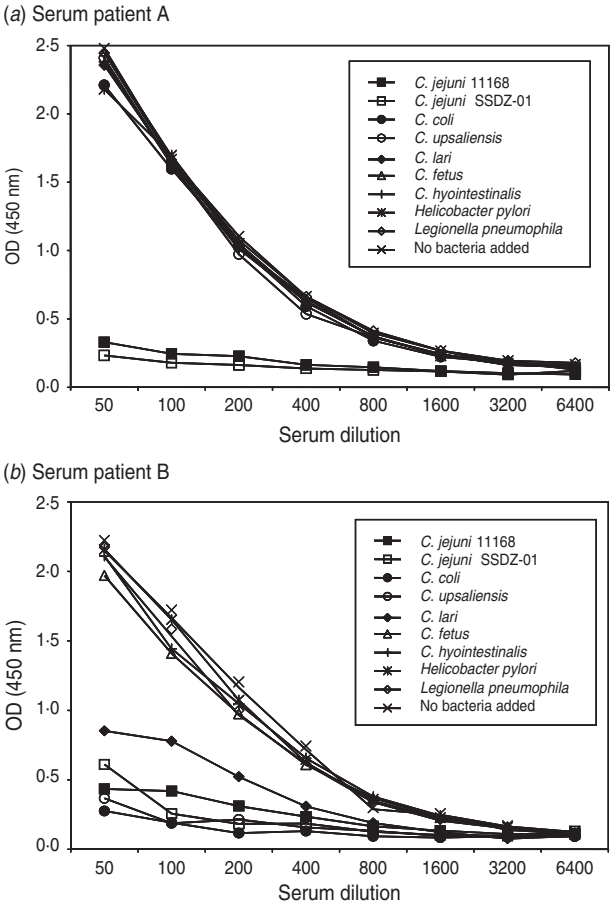

Fig. 1. Specificity of *Campylobacter* IgG reactivity. Serum from seropositive individuals was incubated with bacterial suspensions before testing for IgG antibodies with *C. jejuni* as antigen source. (*a*) Anti-*C. jejuni* reactivity could be reduced by incubation with *C. jejuni* suspensions only, or (*b*) from suspensions with thermophilic *Campylobacter* spp., but not with suspensions of non-thermophilic *Campylobacter* spp., *Helicobacter pylori* or *Legionella pneumophila*. OD, Optical density.

We incubated serum samples of known IgG anti-*Campylobacter* reactivity with bacterial suspensions from various *Campylobacter* spp. and possible cross-reacting species such as *H. pylori* and *L. pneumophila*. During this incubation antibodies directed against the bacteria in the suspension were captured and this led to a decrease in OD value in the *Campylobacter* ELISA. Two patterns of reactivity were observed. In several serum samples reactivity in the *Campylobacter* ELISA could only be inhibited by incubation with *C. jejuni* bacterial suspensions, indicative of specific reactivity to *C. jejuni* (Fig. 1*a*). Other samples had a broader pattern of reactivity. In those samples reactivity in the ELISA could also be decreased by *C. coli*, *C. lari* and *C. upsaliensis* (Fig. 1*b*). There was

https://doi.org/10.1017/S0950268810002359 Published online by Cambridge University Press

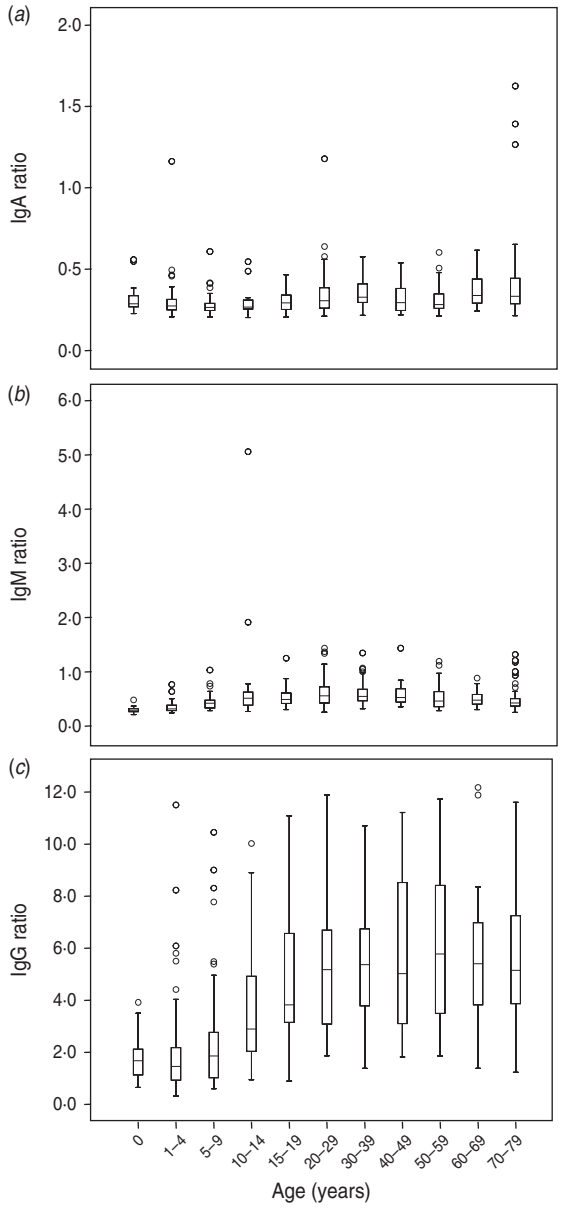

Fig. 2. Levels of IgA, IgM and IgG anti-*Campylobacter* antibodies per age group. Anti-*Campylobacter* antibody levels are expressed as ratios.

no significant reduction in anti-*Campylobacter* reactivity following incubation with non-thermophilic *Campylobacter* spp. such as *C. fetus* and *C. hyointestinalis*, and *H. pylori* or *L. pneumophila*. Together, these results demonstrate that our ELISA system can specifically detect antibody reactivity against thermophilic *Campylobacter* spp.

For all three isotypes IgA, IgM and IgG we investigated the relationship between age, sex, urbanization, and reactivity to *Campylobacter*. IgA ratios gradually increase with age although the ratios remain low (Fig. 2*a*). No significant differences were shown with regard to sex. The degree of urbanization contributes significantly to the model ($P<0{\cdot}01$), slightly higher anti-*Campylobacter* IgA levels were found in rural areas. For IgM, we observed a significant gradual increase in level of IgM antibodies from 0 years to 10–30 years, with a slight decrease observed thereafter (Fig. 2*b*). No significant differences in IgM ratios were seen with regard to sex. The degree of urbanization slightly contributed to the model ($P<0{\cdot}05$) but not in a clear age-dependent manner.

In the analysis of the data for IgG we used a binary mixture model. From Figure 3*a–k*, it can be deduced that with increasing age, the number of seropositive individuals within each age group increases correspondingly. In the 15–19 years age group almost all individuals are seropositive. The number of seropositive individuals within each age group is also shown in Figure 3*l*. This figure clearly shows the increase in seroprevalence during childhood and adolescence. There is an increase with age in both the number of seropositive individuals in any age group, and the mean levels of anti-*Campylobacter* IgG antibodies (Fig. 2*c*).

The relationship between IgG antibody levels and sex and age showed several interesting patterns. The age-related increase of IgG is faster in girls than in boys up to age 20 years at which point it does not increase further. In males IgG ratios increase until about age 35 years and at that age it is significantly higher in males than in females (Fig. 4). For females, this serological pattern parallels the incidence of culture-confirmed symptomatic *Campylobacter* infections in The Netherlands. Males are clearly slower in reaching their maximum anti-*Campylobacter* IgG levels. Above the age of 60 years the levels decrease in males while in females they become higher again. In age groups <25 years we observed higher levels of anti-*Campylobacter* IgG in individuals living in urbanized regions corresponding with a higher incidence (data not shown).

DISCUSSION

Using a highly specific ELISA for detection of antibodies against thermophilic *Campylobacter* spp., we found that the seroprevalence for *Campylobacter* spp. in The Netherlands shows a linear increase during childhood reaching 100% in young adulthood. Our inhibition ELISA experiments demonstrate the specificity of our assay for detecting antibodies against thermophilic *Campylobacter* spp. The assay

https://doi.org/10.1017/S0950268810002359 Published online by Cambridge University Press

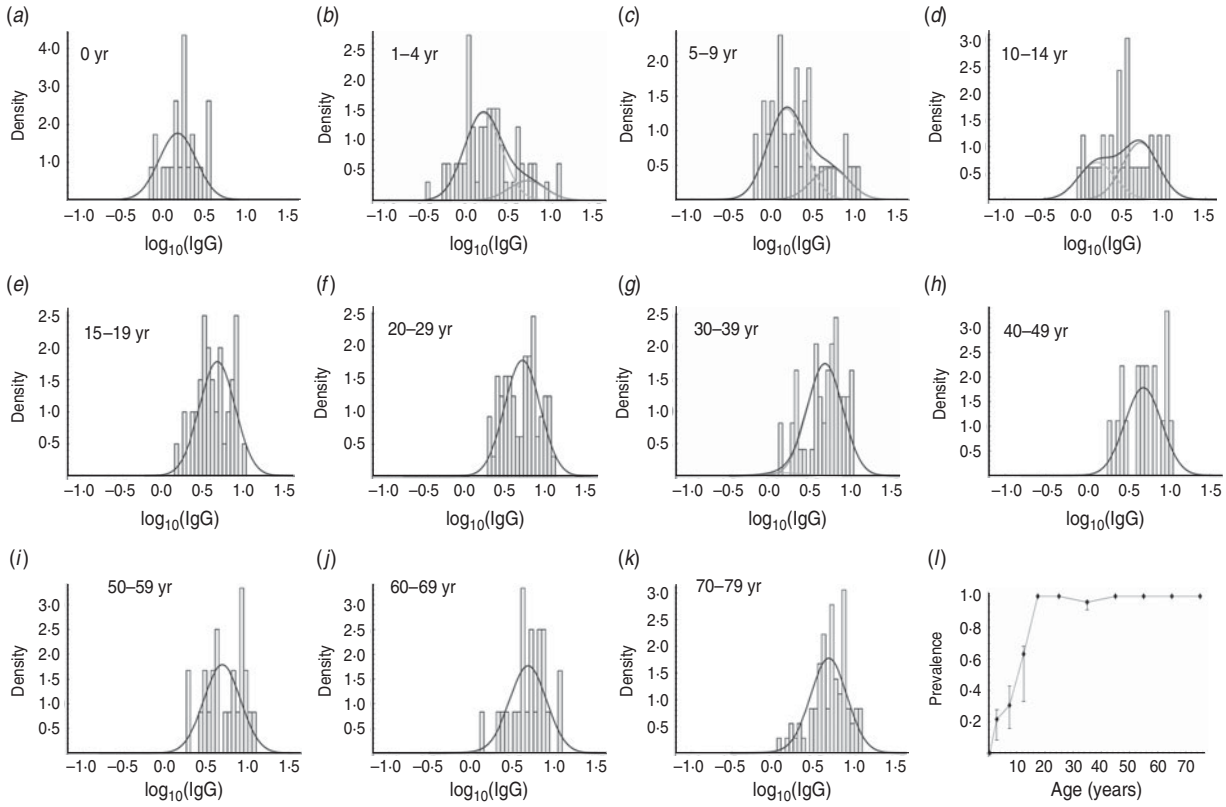

Fig. 3. Frequency distribution of anti-*Campylobacter* IgG ratios in sera from different age groups and fitted components of binary mixture. (*a–k*) Distribution per age group. The line (—) in each figure indicates the distribution of the total group of sera, whereas the histograms indicate the distribution of each component. In (*a*), all individuals are in the component with lower IgG level ('seronegative') and in each consecutive panel, the number of individuals in the component with the higher IgG level ('seropositive') increases. (*l*) Estimated prevalence (number 'seropositive') for each age group.

not only detects antibodies against *C. jejuni*, but is also capable of detecting antibodies directed against other diarrhoea-causing *Campylobacter* spp. The lack of inhibition by incubation with *H. pylori*, *L. pneumophila* and non-thermophilic *Campylobacter* spp. convincingly demonstrates that the measured antibody levels are not caused by cross-reactive or specific reactivity but truly represent antibody reactivity against diarrhoea-causing *Campylobacter* spp.

The binary mixture approach is methodologically attractive. Using this method, it is not necessary to define a cut-off for seronegativity. In many cases, this is theoretically impossible because it can never be stated with certainty that an individual has never had any contact with a pathogen. Furthermore, binary mixture modelling of serological data acknowledges the heterogeneity of the immune response. Most serological tests can be validated for detecting a recent infection, but the binary mixture approach also allows epidemiological studies based on the same serological assays. It is important to bear in mind that cut-off levels for seropositivity intended for detecting a recent acute infection can not be used for epidemiological purposes.

Comparing incidences of gastrointestinal infections based on culture-based surveillance is highly dependent on the organization of the healthcare system. Seroepidemiology in a general population sample eliminates this potential source of bias. In the current study, we tested a random sample of the Dutch population derived from the PIENTER database, which allowed us to draw conclusions at the national level [12]. A potential limitation of this approach is that national serum collections representing the whole population are only available on a very limited scale. This will hamper the widespread use of this relatively easy method to perform epidemiological studies. We have found similar results using serum samples from hospital serum collections (C. W. Ang, unpublished observations). The selection of samples will be facilitated by this finding. Another problem using serological assays to predict infection rates

https://doi.org/10.1017/S0950268810002359 Published online by Cambridge University Press

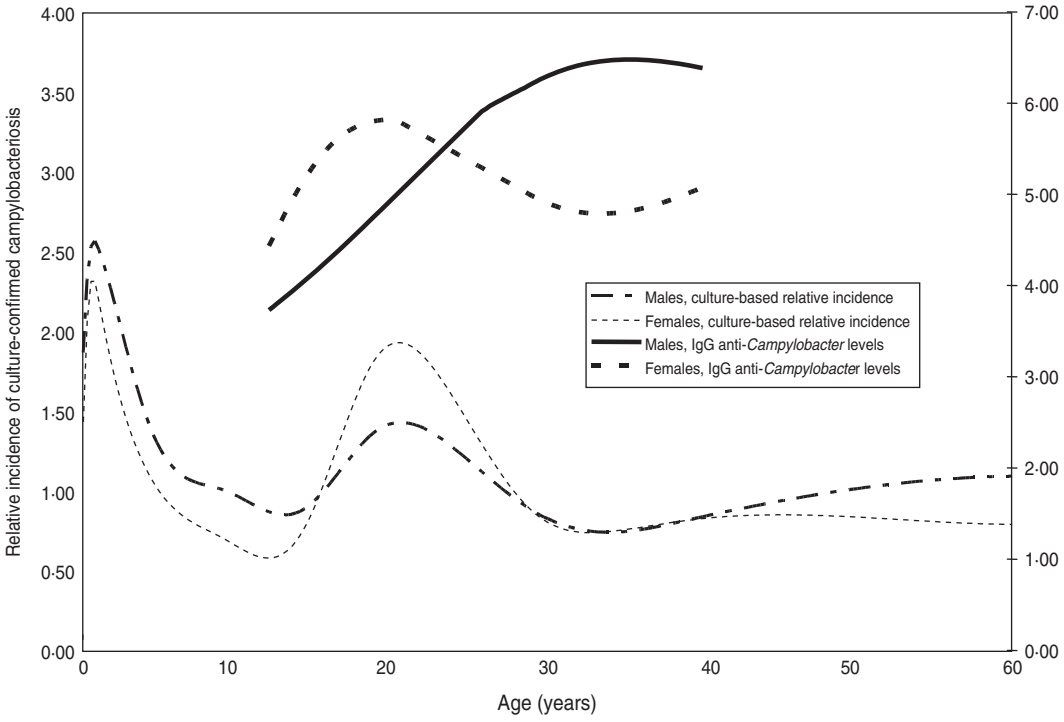

Fig. 4. Estimated levels of anti-*Campylobacter* IgG per age group according to sex, compared with culture-based relative incidence. The estimated age distribution of laboratory-confirmed *Campylobacter* infections in The Netherlands (2003–2006) is plotted for males and females for an average degree of urbanization. The 90% confidence interval is very small and has been omitted. Similarly the fitted average levels have been plotted for the IgG ratio. For the IgG ratio only the age trajectories were plotted that comprise significant differences between males and females, i.e. where the 90% CI (not plotted) did not overlap (12–20 years and 28–40 years).

is inter-laboratory variation, even when using the same assay. Therefore, rigorous validation of tests is needed before drawing any conclusions. Alternatively, all samples should be tested at a single laboratory. When these prerequisites are met, the use of seroepidemiology will allow for direct comparison of the infection pressure between different countries or regions [18].

Comparison of our findings with other studies is not straightforward because it is the first of its design. Belongia *et al*. tested serum samples from rural and non-rural children in the USA [10]. Seropositivity was defined as a 'positive test in 2 or more immunoglobulin classes', where positive was higher than the mean + 2 S.E. of a reference population [19]. Despite their very high cut-off levels for a presumably symptom-free population, they found a gradual increase in seropositivity ranging from 30–40% in 1- to 4-year-olds to 60–85% in 15- to 18-year-olds. In a Danish study, observed percentages were much lower, up to 32% in adults, but this may be due to different criteria for seropositivity [11]. Therefore, despite different approaches, both studies found the same pattern of age-dependent increase of seropositivity in industrialized countries.

In developing countries, the same pattern can be observed although the kinetics of becoming seropositive may be increased. In Africa and South America, there was a gradual increase in total anti-*Campylobacter* immunoglobulin levels in children followed up during the first 2 years of life [20, 21]. In Bangladesh, children acquired IgA, IgM and IgG antibodies against *Campylobacter* early in life, with a fall in IgG levels after the age of 5 years [22].

The most surprising finding is the almost linear increase in IgG seroprevalence during childhood and adolescence. At age 20 years, almost all individuals are seropositive. This implies an average yearly increase of about 5%. It is difficult to provide accurate estimates on the number of infections and re-infections because there are very limited data on immune responses following *Campylobacter* infections in children in industrialized countries. In adults, the half-life of *Campylobacter* IgG has been estimated to be around 2 years (P. F. M. Teunis *et al*., unpublished observations) and it would therefore be unrealistic to

https://doi.org/10.1017/S0950268810002359 Published online by Cambridge University Press

assume that individuals remain seropositive for life. Combined with the observations that in adults anti-*Campylobacter* IgG levels remain high and re-infection in already seropositive individuals does occur [23], the yearly infection rate is likely to be $>5\%$.

The increase in seroprevalence with age, in combination with an increase in level of IgG response is indicative of repeated infections with *Campylobacter*, leading to an antibody response. Based on the incidence of symptomatic *Campylobacter* infections, the majority of these infections are asymptomatic. The possibility remains that the immune system is boosted by dead bacteria in food or fomites. However, living bacteria give much stronger stimulation to the immune system and more severe forms of *Campylobacter* enteritis elicit a stronger humoral immune response [24].

It remains unclear whether the measured immunoglobulin levels can be interpreted as a measure of protection against a symptomatic course of the infection. Well documented cases of re-infection despite pre-existing antibody levels have been described [23]. Our data on the influence of sex and urbanization on antibody levels demonstrate several differences with regard to both parameters. However, they can not be interpreted easily and there is only limited agreement between data derived from culture-based surveillance systems and our serological studies. We were unable to reproduce the findings of Belongia *et al*. with regard to higher anti-*Campylobacter* antibody levels in rural children [10]. In contrast, in The Netherlands, individuals living in urbanized regions have higher anti-*Campylobacter* levels compared to the rural population. These inconsistencies may also depend on different local conditions and definitions of 'rural' *vs*. 'urban' between countries.

In conclusion, this seroepidemiological study indicates that almost all individuals in The Netherlands have multiple *Campylobacter* contacts leading to an antibody response during their lives. Most of these immune response-eliciting events pass with no, or only mild, gastrointestinal symptoms.

ACKNOWLEDGEMENTS

The authors thank Mrs Marijke Maas and Mr Milton Gilbert for excellent technical assistance.

DECLARATION OF INTEREST

None.

REFERENCES

1. **Havelaar AH, *et al.*** Health loss by microbial agents in food. In: *Future Explorations of Public Health, National Compass of Public Health*. Bilthoven: RIVM, 2008.
2. **Havelaar AH, *et al.*** Health burden in The Netherlands due to infection with thermophilic *Campylobacter* spp. *Epidemiology and Infection* 2000; **125**: 505–522.
3. **De Wit MA, *et al.*** Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and etiology. *American Journal of Epidemiology* 2001; **154**: 666–674.
4. **Wheeler JG, *et al.*** Study of infectious intestinal disease in England: rates in the community, presenting to general practice, and reported to national surveillance. The Infectious Intestinal Disease Study Executive. *British Medical Journal* 1999; **318**: 1046–1050.
5. **Van Pelt W, *et al.*** Laboratory surveillance of bacterial gastroenteric pathogens in The Netherlands, 1991–2001. *Epidemiology and Infection* 2003; **130**: 431–441.
6. **Samuel MC, *et al.*** Epidemiology of sporadic *Campylobacter* infection in the United States and declining trend in incidence, FoodNet 1996–1999. *Clinical Infectious Diseases* 2004; **38** Suppl 3: S165–174.
7. **Rajan DP, Mathan VI.** Prevalence of *Campylobacter fetus* subsp. *jejuni* in healthy populations in southern India. *Journal of Clinical Microbiology* 1982; **15**: 749–751.
8. **Glass RI, *et al.*** Epidemiologic and clinical features of endemic *Campylobacter jejuni* infection in Bangladesh. *Journal of Infectious Diseases* 1983; **148**: 292–296.
9. **Coker AO, *et al.*** Human campylobacteriosis in developing countries. *Emerging Infectious Diseases* 2002; **8**: 237–244.
10. **Belongia EA, *et al.*** Diarrhea incidence and farm-related risk factors for *Escherichia coli* O157:H7 and *Campylobacter jejuni* antibodies among rural children. *Journal of Infectious Diseases* 2003; **187**: 1460–1468.
11. **Linneberg A, *et al.*** IgG antibodies against microorganisms and atopic disease in Danish adults: the Copenhagen Allergy Study. *Journal of Allergy and Clinical Immunology* 2003; **111**: 847–853.
12. **De Melker HE, Conyn-van Spaendonck MA.** Immunosurveillance and the evaluation of national immunization programmes: a population-based approach. *Epidemiology and Infection* 1998; **121**: 637–643.
13. **Allos BM.** *Campylobacter jejuni* infections: update on emerging issues and trends. *Clinical Infectious Diseases* 2001; **32**: 1201–1206.
14. **Ang CW, *et al.*** Validation of an ELISA for the diagnosis of recent *Campylobacter* infections in Guillain-Barré and reactive arthritis patients. *Clinical Microbiology and Infection* 2007; **13**: 915–922.
15. **Ramsay JO.** Monotone regression splines in action. *Statistical Science* 1988; **3**: 425–441.
16. **Harrell Jr. FE.** *Regression Modeling Strategies*. New York: Springer-Verlag, 2001.
17. **Gilks WR, *et al.*** *Markov Chain Monte Carlo in Practice*. London: Chapman and Hall, 1996.

18. **Simonsen J, *et al.*** Seroepidemiology as a tool to study the incidence of *Salmonella* infections in humans. *Epidemiology and Infection* 2008; **136**: 895–902.
19. **Blaser MJ, Duncan DJ.** Human serum antibody response to *Campylobacter jejuni* infection as measured in an enzyme-linked immunosorbent assay. *Infection and Immunity* 1984; **44**: 292–298.
20. **Martin PM, *et al.*** Immune response to *Campylobacter jejuni* and *Campylobacter coli* in a cohort of children from birth to 2 years of age. *Infection and Immunity* 1989; **57**: 2542–2546.
21. **Figueroa G, *et al.*** Prospective study of *Campylobacter jejuni* infection in Chilean infants evaluated by culture and serology. *Journal of Clinical Microbiology* 1989; **27**: 1040–1044.
22. **Blaser MJ, *et al.*** *Campylobacter jejuni*-specific serum antibodies are elevated in healthy Bangladeshi children. *Journal of Clinical Microbiology* 1985; **21**: 164–167.
23. **Baqar S, *et al.*** *Campylobacter jejuni* enteritis. *Clinical Infectious Diseases* 2001; **33**: 901–905.
24. **Rollwagen FM, *et al.*** Killed *Campylobacter* elicits immune response and protection when administered with an oral adjuvant. *Vaccine* 1993; **11**: 1316–1320.