

## Opinion

# Campylobacter and fluoroquinolones: a bias data set?

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### Summary

There is no universally accepted standard method for the isolation of *Campylobacter* spp. and it is considered that currently available isolation media are not yet optimal for the recovery of *Campylobacter* spp. from a range of sample types. Almost all methods incorporate antibiotics into the isolation media to inhibit growth of other bacteria within the sample. It is established that the incorporation of such antibiotics into isolation media will inhibit the growth of some *Campylobacter* spp. as well as other bacteria.

The results of the use of such suboptimal isolation methods are that the isolates which 'survive' the isolation procedure will be those which: (i) are able to 'out compete' the rest of the bacteria in the sample, i.e. they are able to grow faster; (ii) are resistant to the antibiotics used in the isolation media; and (iii) are randomly selected by the laboratory technician as being a 'typical' *Campylobacter* spp. It is clear that such a procedure is intrinsically biased and will mean that species resistant to the antibiotics used in the media will be isolated. This introduces real doubt that the bacteria isolated are truly representative of those initially found on the sample.

It is also becoming clear that *Campylobacter* spp. are rather difficult to isolate as pure cultures and many are in fact mixtures of more than one strain. Again this introduces great uncertainty as to the prevalence and distribution of respective species from the different sample types. This is especially true when considering isolation of *Campylobacter* spp. causing disease in man as there is no certainty that the selected isolate is that which was responsible for disease.

The incorporation of antibiotics into the isolation media not only introduces the issue of species bias but perhaps more importantly exposes the *Campylobacter* spp. to a cocktail of antibiotics thereby providing the potential for them to 'switch on' antibiotic resistance mechanisms. It might be argued that this has always been the case for isolation of *Campylobacter* spp., however, we know that the antibiotic cocktails used in media over the last 10 years have changed and indeed there was a time when the filtration protocol which didn't use antibiotics was more widely used. As most reports in the literature do not state what methods were used to isolate *Campylobacter* spp. it is not possible to quantify any relationship between antibiotics used in the isolation media and susceptibility data.

An approved method for *Campylobacter* susceptibility testing was not available until May 2002, all data generated prior to this date will have been generated using non-standard methods. As tremendous variability in the reproducibility data for *Campylobacter* spp. was observed during the development of the standard agar dilution susceptibility method, data generated with disk diffusion and broth microdilution methods must be considered with caution. It has been shown that, compared with the conventional agar dilution method, the E-test tends to give rise to lower minimal inhibitory concentrations (MICs) for sensitive strains and higher MICs for resistant strains.

There are no recommended antibiotic breakpoint concentrations for *Campylobacter* spp. A breakpoint is used to separate sensitive from resistant strains of bacteria and is thus crucial to any discussion of antibiotic resistance. This discussion is further complicated by introduction of the terms microbiological and clinical breakpoints. While a microbiological breakpoint can be a useful parameter with regard to identifying resistance factors it cannot on its own be used to predict whether that bacteria will respond to treatment from an appropriate antibiotic. Predicting clinical response is a function of the clinical breakpoint which considers the pharmacokinetic profile of the antimicrobial compound, i.e. the concentration of the antimicrobial compound in the body and the MIC.

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**The National Committee for Clinical Laboratory Standards (NCCLS) uses microbiological, pharmacokinetic and clinical data to establish breakpoints, without such considerations it is not possible to consider what is truly clinically sensitive and resistant. There are no reported studies that have systematically determined appropriate breakpoints for *Campylobacter*, there are data however, which relate MICs to clinical outcome. It is without dispute that microbiological resistance in *Campylobacter* spp. occurs as a result of mutation in the *gyrA* gene with single point mutations most frequently causing a four- to eightfold shift in the MIC. What is also clear is that if a high enough concentration of antimicrobial relative to MIC of the infecting organism can be achieved not only will the parent organism be killed but also the 'resistant' mutant. Considering the above and the concentrations of ciprofloxacin achieved in the gastrointestinal tract it is not surprising that clinical cure can be demonstrated for organisms with an MIC of 32 µg ml<sup>-1</sup>.**

## Introduction

*Campylobacter* are widespread in several animal species and a cause of gastro-intestinal infection in man. This disease is self-limiting and does not normally require therapy unless the individual is immunosuppressed or the infection is non-intestinal (Nachamkin, 1993).

Fluoroquinolones show *in vitro* activity against *Campylobacter* spp. and have been used to treat gastroenteritis and in prophylaxis for travellers' diarrhoea (Fliegelman *et al.*, 1985; DuPont *et al.*, 1987). It is evident that there is not always a clear relationship between MIC (minimum inhibitory concentration) data, microbiological outcome and clinical outcome following fluoroquinolone therapy. This can, in part, be attributed to a number of issues which are yet to be subject to rigorous scientific debate. This review contributes to that debate and considers isolation of *Campylobacter* spp., from human and animal sources as well as susceptibility testing and interpretation of *in vitro* data as an indicator of clinical response to fluoroquinolone therapy. The fundamental question being posed is whether currently used isolation procedures bias our understanding of the role of *Campylobacter* spp. in disease.

## Isolation of *Campylobacter* spp.

### *Isolation procedures*

Fundamental to susceptibility testing of any group of organisms is a requirement to first isolate the organism responsible for infection. Current NCCLS

(<http://www.nccls.org>) documents clearly state that when the specimen contains mixed growth or normal flora, in which the organisms probably bear little relationship to the infectious process being treated, susceptibility tests are often unnecessary, and the results may be misleading (NCCLS, 2000, M7–A5; NCCLS, 2002, M31–A2). The reason is that there may not be a sound clinical basis for the treatment chosen if the organism isolated is not representative of the organism causing disease. The issue with *Campylobacter* susceptibility testing is rather complex in that isolates are obtained from various sources, animals, foods including drinking water, from the environment and from subclinical and clinical infection in man. Irrespective of the origin of the sample it is crucial to ensure that no bias is introduced into the population to be tested as a result of the isolation procedure. It is clear from the literature that this important issue has not been addressed.

### *Is there a standard approach?*

Corry *et al.* (1995), in an extensive review of the literature, concluded that there is no generally accepted 'standard' method of isolating *Campylobacter* spp. Indeed in the conclusion to the review the authors commented that media for isolating campylobacters from faeces, food and water were not yet optimal and there is no consensus concerning the best media and methods. On (1996) similarly stated that significant improvements in methods for detecting *Campylobacter* spp. are required. The media used for isolating campylobacters from food and water has been largely derived from those developed for the isolation of campylobacters from faeces. In keeping with techniques developed for isolation of other pathogens, liquid enrichment and pre-enrichment media have been used. One of the conclusions of On (1996) is that the isolation procedure for *Campylobacter* spp. introduces bias into the data set. The campylobacter literature is full of comparative data comparing and contrasting antimicrobial susceptibility patterns from organisms isolated from human and animal sources, although there is no reference to the different methodologies used in the respective laboratories. It must be understood that the approaches to isolation of *Campylobacter* spp. from man will be fundamentally different to those from animal sources. In cases of diarrhoea in man the numbers of *Campylobacter* spp. in the sample will be significant whereas in veterinary laboratories there will be a much higher ratio of non-campylobacter to campylobacter isolates and so the isolation procedures will be amended accordingly. The enrichment phase is therefore more important for animal samples, however, this will provide an opportunity for the faster growing *Campylobacter* strains to dominate rather than being a reflection of the actual balance of isolates

naturally present in the initial sample. It will be argued that this bias must consequently be taken into consideration when comparing human and animal data. Besides a variety of selective agents incorporated into the media, almost all of which are antimicrobials, media for campylobacters usually contain ingredients to neutralize the toxic effects of substances formed in the presence of oxygen and light (Corry *et al.*, 1995). In addition almost all workers have found it necessary to incubate plates in an atmosphere of 5–7% oxygen, 10% carbon dioxide, up to 5% hydrogen with the balance as nitrogen. More recent work, however, has suggested that optimal incubation atmospheres will vary according to species (Annable *et al.*, 1997) although no systematic investigation of the effect of atmosphere during incubation of media has been carried out (Corry *et al.*, 1995).

Antimicrobials in selective media developed for campylobacters have been chosen on the basis of those to which test strains were resistant and those most effective in inhibiting competitive flora. At least 17 different single antimicrobials have been used (cephalothin, cephalosporin, cephazolin, cefsulodin, cephalixin, cefoperazone, trimethoprim, polymyxin B, colistin, vancomycin, teicoplanin, rifampicin, novobiocin, bacitracin, cycloheximide, actidione, amphotericin, nystatin) either singly or more often in combination, including five different cephalosporins. Corry *et al.* (1995) made the point that with few exceptions the strains used for susceptibility testing against antimicrobials to be used for inclusion in media formulations would have first been isolated using antimicrobial-containing selective media and so the possibility of missing strains sensitive to the antibiotic used in the selective media will have been perpetuated. Indeed there is evidence that some strains of *C. coli* and even a few strains of *C. jejuni* are likely to have been missed because of their sensitivity to cephalothin (Ng *et al.*, 1985; 1988; Brooks *et al.*, 1986). More recent work has indicated that other species of campylobacter, *C. upsaliensis*, of importance in human intestinal disease will not be isolated by the usual selective agar because of their susceptibility to the antibiotics used in most media (Walmsley and Karmali, 1989; Goossens *et al.*, 1990).

#### *Does antibiotic use in isolation media introduce bias?*

It is incorporation of antimicrobials into the media that has probably the greatest significance with regard to introducing bias into the isolation procedure. Indeed other workers have identified that the procedure for isolation of *C. jejuni* and *C. coli* uses selective agents that will inhibit other species of campylobacter (Goossens and Butzler, 1992; ACMSF, 1993; Lawson *et al.*, 1998). Lastovica and Le Roux (2000) only began to isolate *Campylobacter upsaliensis*, *Campylobacter concisus*, *Campylobacter curvus*,

*Campylobacter rectus*, *Campylobacter sputorum* biovar *sputorum* with the development of the 'Cape Town protocol' which does not use any antibiotics in the selective medium. Lawson *et al.* (1997) clearly showed that some strains of *Campylobacter upsaliensis* and *Campylobacter helveticus* were susceptible to levels of cefoperazone incorporated into selective media and consequently failed to grow or were detected with reduced sensitivity in the presence of antibiotic. Similarly Aspinall *et al.* (1993) were unable to detect 18% of *C. upsaliensis* strains seeded at a level of  $10^5$ – $10^6$  cfu g<sup>-1</sup> faeces with an agar containing 8 mg l<sup>-1</sup> of cefoperazone. While the focus of our attention is directed at *C. jejuni* and *C. coli* it is necessary to consider other campylobacter such as *C. upsaliensis* which contributes significantly to the total *Campylobacter* isolation rates in diarrhoea and is now being considered as being important as a cause of human enteric infection (Bourke *et al.*, 1998). *Campylobacter upsaliensis* represented 12% of all *Campylobacter* isolates in the study of Goossens *et al.* (1990). Bourke *et al.* (1998) reviewed other data on isolation rates of *C. upsaliensis* and suggested that *C. upsaliensis* may account for over 10% of all faecal *Campylobacter* isolates and in infants and young children it may be closer to 20%. As *C. upsaliensis* is sensitive to nalidixic acid and, usually to cephalothin (Bourke *et al.*, 1998) the presence of such antibiotics in the culture medium may well account for the suboptimal identification of this organism in clinical specimens. If indeed it is present at such high levels it is interesting to speculate why there has not been any apparent development of resistance to fluoroquinolones.

Moore and Murphy (2000) reported the inability of CAT (cefoperazone amphotericin teicoplanin) selective medium to grow a significant number of thermophilic *Campylobacter* spp. which had initially been isolated from foodstuffs. More than 10% of 245 isolates failed to grow on CAT medium under optimal conditions after isolation. It was considered that this was due to the antibiotics used and Moore and Murphy (2000) concluded that the choice of isolation medium may influence the relative distribution of *Campylobacter* spp. and phenotypes recovered.

The review of Corry *et al.* (1995) clearly showed that use of different media will bias the isolation of *Campylobacter* spp., it also commented that none of the described media utilized indicator systems for the identification of presumptive campylobacter colonies, unlike the case for other pathogens. The net effect being that isolation is often a result of experienced workers being able to recognize the organism by the typical appearance of isolated colonies. Corry *et al.* (1995) made the point that campylobacter colonies can be atypical. Any procedure that relies on individual expertise will inevitably introduce bias especially when data needs to be considered nationally and internationally.

The nature of the bias arising from use of selective media was clearly illustrated in a large scale UK survey of *Campylobacter* species in man where samples were screened by a PCR-enzyme-linked immunosorbent assay after first being cultured by regional laboratories (Lawson *et al.*, 1999). *Campylobacter jejuni* and *C. coli* represented the largest proportion of PCR-positive samples. There was congruence between PCR and culture for 77.5% of the 529 positive samples, however, 12.9% were found to be positive only by PCR and 9.6% were positive only by culture. Although reasons were put forward to explain the differences the study found more positive samples by PCR-ELISA than by culture alone. The authors attributed this to the ability of PCR to detect cells in metabolic states that are less amenable to culture on selective media. Eleven cases of gastroenteritis were attributed to *C. upsaliensis*, three cases to *C. hyointestinalis* and one to *C. lari*. This represented the highest incidence to date of reported cases of *C. upsaliensis*, only two of which were positive by culture and in neither case was it attributed to *C. upsaliensis*. The authors also reported that in 19 samples there was evidence of mixed infection with *C. jejuni* and *C. coli*, this was not apparent from culture. Seven of these cases were from a laboratory which had the ability to identify isolates to the species level and which reported that five were *C. jejuni* and two *C. coli*. It was therefore considered that only the predominant colony type had been selected for identification, once again illustrating the potential for introducing bias into the data. Richardson *et al.* (2001) detected a 7.5% frequency of coinfection of *Campylobacter* in 53 cases of human infection. This was similar to that of Ruberg *et al.* (1998) who sampled 42 patients but only a quarter of that determined by Kramer *et al.* (2000) in a study looking at the frequency of contamination of meats. The differences may, however, reflect the small numbers of patients studied. These authors concluded that mixtures of *Campylobacter* species (and serotypes) in human infections might be more common than was heretofore assumed. If this is indeed true then it introduces serious concerns as to whether enrichment culture techniques are able to isolate those organisms responsible for infection. This also raises the question whether all isolates from an infectious incident are indeed implicated in the disease process. It is clear that if the respective components of a mixed culture have different growth rates then that with the faster growth is likely to dominate in a selective enrichment culture. This also has great significance with regard to susceptibility testing if indeed there is no certainty that the tested isolates are those solely and directly responsible for the infection. The diversity of *Campylobacter* isolated from retail poultry carcasses was studied by Dickins *et al.* (2002) using a direct isolation procedure that eliminated the pre-enrichment step. The authors acknowledged that

the long pre-enrichment stage provides an opportunity for rapidly growing strains to be selected and overgrow slower-growing strains, whereas a direct plating procedure, albeit utilising antibiotics, would be expected to provide a better representation of the population present on a carcass. They accepted that the direct plating procedure may cause injury to some *Campylobacter* spp. and that their data was probably an underestimate of the total diversity on a carcass. Despite these reservations the authors found more than one strain of *Campylobacter* spp. on 67% of infected carcasses and up to six strains were found on a single carcass. They acknowledged that the chicken may have been superinfected with more than one strain in the brooder house, or contaminated post-slaughter. Irrespective of this fact what is clear is that to get a true measure of the diversity then there will need to be changes to the standard procedure used for isolating *Campylobacter* spp. Newell *et al.* (2001) also clearly showed that the method of recovery from poultry carcasses will influence the subtypes of strains observed. They used two recovery techniques, direct plating and prior enrichment, enrichment being considered the optimal recovery method for campylobacter's under stress conditions, such as from poultry carcasses. In several cases strain types were recoverable by direct plating but not by enrichment, suggesting that enrichment will preferentially select certain strains. This selection was not so obvious in the cultures from caecal contents, presumably because the organisms were present in larger numbers and in a relatively unstressed state. The authors concluded that further investigations are required in this area and that multiple techniques may be necessary to obtain a clear picture of the subtypes present in any mixed campylobacter population. Miller *et al.* (2000) have shown that even the selection of single colonies of *Campylobacter* spp. can be misleading. In a rather elegant study they developed two sets of *Campylobacter* shuttle vectors containing either the green fluorescent protein (gfp), yellow fluorescent protein (yfp) or cyan fluorescent protein (cfp) reporter gene. Experiments with the yellow fluorescent and cyan fluorescent *C. jejuni* transformants suggested that aggregates containing two or more strains of *C. jejuni* may be present in an enrichment broth culture. Colonies arising from these aggregates would be heterologous in nature. It can therefore be seen that isolation of a 'pure' culture of *C. jejuni* by selecting single colonies, from an environmental sample may not always yield a single strain.

Borck *et al.* (2000) concluded that that the method routinely used for surveillance in turkeys in Denmark was suboptimal. They additionally pointed out that sublethally damaged cells are often more sensitive to the selective antimicrobial agents used in traditional culture approaches and that enrichment steps need to be devel-

oped as being specific to the sample type under investigation. This arose from the fact that recoveries of campylobacters from turkey meat and neck skin differed depending on the growth media used.

### Possible implications of use of antimicrobials in isolation media

#### *Can antimicrobials in the media affect the susceptibility test result?*

It is clear that use of antimicrobials in isolation media is one of the factors that will bias the frequency and nature of campylobacter isolation. There are, however, other effects mediated by the use of antimicrobials in isolation media that are rarely discussed. In a session at the 2002 American Society for Microbiology General Meeting, Fedorka-Cray publicly questioned the impact of using antimicrobials in isolation media (Fedorka-Cray, 2002). Intuitively one might expect that any use of antimicrobials in isolation media will impact upon the very organisms that are being isolated. Indeed when submitting MIC (minimum inhibitory concentration) data to regulatory authorities with regard to antimicrobial safety studies it is conditional that MICs must be generated in isolates that have not been exposed to antibiotics for at least three months prior to isolation. It thus seems rather untoward that great scientific weight is being placed on MIC susceptibility data that has been generated from isolates, which have been exposed to antibiotic cocktails as part of the isolation process.

While reviewing the Campylobacter MIC susceptibility literature it is interesting to note that relatively few authors detail the isolation procedures used and as such it is difficult to generate data on which antibiotics were actually used as selective agents. This in part reflects laboratory structures as it is normally local laboratories that isolate the campylobacter strains and then submit to regional or national centres where susceptibility testing is carried out. This clearly separates the two laboratory functions of isolation and test and has maybe served to divert attention away from asking such fundamental questions as to whether the isolation process could influence the susceptibility test result.

#### *$\beta$ -lactam and quinolone susceptibility can be affected in E. coli*

It is clear that the use of antimicrobials in the isolation media has the opportunity to have a profound effect on MIC data. The most common class of antimicrobial used in campylobacter media is the  $\beta$ -lactam class of antibiotics, initially cephalothin was used although this has now largely been replaced by cefoperazone. In 1983 Moyed and Bertrand first identified the *hipA* gene in *E. coli* and

showed it to be responsible for the persistence of cells in the presence of antimicrobials that inhibit peptidoglycan synthesis including of course the  $\beta$ -lactam antibiotics. Scherrer and Moyed (1988) further reported that *hipA* was not only implicated in persistence to  $\beta$ -lactams but also to selective inhibition of DNA synthesis. It is thus clear that *E. coli hipA* mutants, selected following exposure to  $\beta$ -lactam antibiotics, are also killed less effectively by nalidixic acid (Scherrer and Moyed, 1988). These and other properties of *hipA* mutants suggested the possibility of a common pathway for killing by  $\beta$ -lactams and quinolones (Scherrer and Moyed, 1988). Wolfson *et al.* (1990) confirmed this to be the case and additionally demonstrated *hipQ*, a new mutant locus, which was responsible for the pattern of reduced killing by norfloxacin and ampicillin. These findings indicate that there is an overlap in killing of *E. coli* by DNA gyrase A-subunit antagonists and cell wall active agents. Despite *hipQ* and *hipA* resulting in reduced killing by quinolones and  $\beta$ -lactams Wolfson *et al.* (1990) showed them to be distinct and responsible for different mechanisms of killing.

What is clear is that in *E. coli*, *hipA* and *hipQ* are selected for in the presence of a  $\beta$ -lactam antibiotic resulting in isolates with not only increased MICs to the  $\beta$ -lactam but also to quinolones. As has been illustrated  $\beta$ -lactam antibiotics are widely used in campylobacter isolation media and in the absence of data to the contrary we cannot conclude that similar mechanisms are not found in *Campylobacter* spp. *HipA* is not found in *Campylobacter* spp. although there does not appear to be any database entry at NCBI for *hipQ*. It is not known, however, if there are any similar mechanisms in campylobacter that could result in susceptibility bias in isolates arising from the incorporation of antibiotics into the isolation media.

#### *Efflux pumps – may they play a role?*

Similar results could arise from selection for multidrug efflux pumps arising from use of selective agents. Lin *et al.* (2002) have recently added to our limited knowledge of drug efflux mechanisms in *C. jejuni* by characterizing an efflux pump, designated *CmeABC*, that contributes to multidrug resistance in *C. jejuni*. Such efflux systems not only contribute to the intrinsic resistance to fluoroquinolones in *Campylobacter* spp. but also are able to confer acquired resistance to fluoroquinolones by over expression of the efflux proteins (Poole, 2000) or by synergetic interplay with non-efflux fluoroquinolone resistance mechanisms, such as *gyrA* mutations (Lomovskaya *et al.*, 1999; Oethinger *et al.* 2000). These efflux mechanisms are not necessarily specific to antimicrobial class and so can be turned on by other classes of antimicrobial compound as is the case for the

*CmeABC* system. Whereas the resistance issues in *Salmonella* spp. are a separate issue it is perhaps interesting to consider the recent work of Giraud *et al.* (2000) who suggested that efflux mechanisms may appear early compared to mutations in *gyrA* and could thus be responsible for the first decrease in susceptibility to fluoroquinolones. Further selective pressure by antibiotics, in the case of Giraud *et al.* (2000) this was considered to be therapeutic antibiotics, but it could equally be as incorporated into selective media, may trigger the selection of *gyrA* mutants and the appearance of fully resistant phenotypes. In a recent paper Piddock *et al.* (2002) described a ciprofloxacin-resistant, nalidixic acid-susceptible mutant of *Staphylococcus aureus*. This combination of fluoroquinolone resistance and nalidixic acid sensitivity has not been previously described for *S. aureus*. Moniot-Ville *et al.* (1991) identified a similar phenotype in *E. coli* which was attributed to reduced fluoroquinolone uptake and a *gyrA* mutation. Piddock *et al.* (2002) failed to detect any mutations within the quinolone resistance-determining regions of the genes coding for topoisomerase IV and DNA gyrase, indicating that efflux mechanisms might be involved.

The contribution of efflux mechanisms to resistance development is clearly complex and our understanding of this phenomenon is still at an early stage. It is clear, however, that compounds other than fluoroquinolones will impact upon this mechanism and incorporation of antimicrobials into culture medium has the potential to select for resistance to compounds other than the selective agents.

#### Other mechanisms?

A link between  $\beta$ -lactam antibiotics and fluoroquinolones in *Streptococcus pneumoniae* has recently been suggested by Carsenti *et al.* (2002). Working with penicillin-susceptible and -resistant isolates they showed that the rate of acquisition of resistance to fluoroquinolones is strongly related to alteration of susceptibility to penicillin. In particular they demonstrated that for isolates with penicillin MICs  $>0.25 \mu\text{g ml}^{-1}$  it required significantly fewer passages in subinhibitory concentrations of ciprofloxacin to develop resistance compared to isolates with penicillin MICs  $<0.25 \mu\text{g ml}^{-1}$ . Higgins *et al.* (2002) have presented data from more than 3000 Gram-negative isolates showing that the relationship between ciprofloxacin resistance and resistance to unrelated drugs is statistically significant. They didn't investigate the resistance mechanisms or the relatedness of isolates within a species and thus didn't attempt to speculate at reasons for the relationship. They did, however, comment that ciprofloxacin resistance probably followed multidrug resistance, and that ciprofloxacin therapy might have been used when other therapeutic options were compromised because of resistance.

## Susceptibility testing

The fastidious growth requirements and fragility of *Campylobacter* species present significant challenges to the conduct of antimicrobial susceptibility tests. The fastidious nature of campylobacter growth is reflected by the number of variations in media, atmosphere, incubation temperature, duration and inoculum between methods, making direct comparison difficult. What is beyond debate is that an approved method for *Campylobacter* susceptibility testing was not available until May 2002 when NCCLS published M31-A2 (NCCLS, 2002). Despite this many publications claim that susceptibility testing of *Campylobacter* spp. was carried out in accordance with NCCLS methodology. M31-A2 clearly states that agar dilution is the method of choice for testing *Campylobacter* isolates. Disk diffusion testing has been shown not to be valid for testing *Campylobacter jejuni* and related species. Broth microdilution studies are in progress but as yet cannot be recommended.

It is clear that the vast majority of authors have not even considered the principles laid down in the NCCLS Guideline M37-A2 with regard to how one evaluates the utility of an appropriate method for determining its susceptibility to a test antimicrobial compound.

In the manual of Clinical Microbiology published by The American Society for Microbiology (Anonymous, 1995) there is a clear reminder that susceptibility tests for *Campylobacter* spp. are not standardized. The text indeed goes further and notifies the reader that,

'the literature contains some variability in the susceptibility data reported'.

A similar precautionary note is provided by Kays and Graff (2002) not in relation to *Campylobacter* but to the fastidious *Streptococcus pneumoniae*. The authors caution that,

'When evaluating fluoroquinolone activity and pharmacodynamics against this organism, clinicians must be aware that MIC testing methodology may have a significant impact on results.'

Although susceptibility testing of *Streptococcus pneumoniae* presents its own problem the principles behind the above statement are equally true for *Campylobacter* spp. Assuming that there is a standardized susceptibility test method it is crucial that the resultant data is interpreted in an appropriate manner.

## Interpretation of the data

### *Do we really understand what we mean by a breakpoint?*

In addition to not having a standardized method for susceptibility testing, scientifically determined microbiological or clinical breakpoints have not been determined for campylobacter of human or veterinary origin. This has led

to much confusion in the literature when describing isolates as being resistant or susceptible. Clearly much of the literature has simply taken into account an assumed microbiological breakpoint. Hayward *et al.* (1999) assumed ciprofloxacin interpretive breakpoints for *C. jejuni* to be similar to the *Enterobacteriaceae* and applied a sensitive breakpoint as  $\leq 1 \mu\text{g ml}^{-1}$  and resistant as  $\geq 4 \mu\text{g ml}^{-1}$ , as did Endtz *et al.* (1991). Huysmans and Turnidge (1997) proposed a set of interpretive zone criteria for a range of antimicrobials including ciprofloxacin against thermophilic campylobacters. They supported a tentative resistant breakpoint of  $4 \mu\text{g ml}^{-1}$  for ciprofloxacin but apart from two strains of the intrinsically resistant *C. lari* all other strains used in the study were susceptible. The study was thus limited in its outcome.

In a review of quinolone resistance and *Campylobacter* spp. Piddock (1995) referred to

'the recommended breakpoint concentration of fluoroquinolones as  $1\text{--}4 \text{ mg l}^{-1}$ ' without detailing who had made the recommendation. Piddock now realizes this 'There are no recommended antibiotic breakpoints concentrations (or an agreed susceptibility testing method) for *Campylobacter* spp.' (Piddock *et al.* 2000).

Clearly there are no reported studies that have systematically determined appropriate breakpoints for *Campylobacter*. It thus becomes difficult to give any credence to data considering isolates as resistant when having an MIC of  $0.5 \mu\text{g ml}^{-1}$ .

#### *The importance of clinical breakpoints*

It is important to establish that NCCLS uses microbiological, pharmacokinetic and clinical data to establish breakpoints (NCCLS, 2000; M7-A5) and without such considerations it is not possible to consider what is truly clinically sensitive and resistant.

Dudley and Ambrose (2000) elegantly highlighted these important issues. The MIC distribution pattern for a large number of microorganisms often enables identification of two or more populations of microorganisms that can be differentiated by the presence or absence of resistance factors. 'Susceptible' and 'resistant' MIC breakpoints can thus be established to differentiate these populations. The second consideration is the pharmacokinetic profile of the antimicrobial compound and finally the resulting clinical outcome. Dudley and Ambrose (2000) highlighted the challenge of combining the above to produce a single susceptibility/resistance breakpoint and asked the question, 'should a breakpoint detect resistance or predict the antimicrobial effects of a drug in a patient when the drug is administered as a normal dose'. These are two fundamentally different functions, it would appear that the considerable disagreement that often emerges in selecting breakpoints stems from a failure to recognize this differ-

ence and hence a desire to combine all information into a single function (Dudley and Ambrose, 2000). Dudley and Ambrose (2000) suggested that the dual objective for breakpoints to detect resistance/predict outcome will continue to fail in many circumstances, and continue to serve as a source of confusion among clinicians, clinical microbiologists, regulators and researchers. Moreover, in an age in which clinicians manage infections by empiric therapy of a patient syndrome, the need to consider the distribution of MICs as well as drug exposure when administering safe doses in target populations must be taken into account while establishing MIC breakpoints for susceptibility.

#### *What does the data suggest?*

It is clear that such data sets are not available for the fluoroquinolones and *Campylobacter* spp., notwithstanding this fact there are data which relate MICs to clinical outcome. Segreti *et al.* (1992) showed that in a clinical trial of ciprofloxacin for therapy of acute diarrhoea, two subjects infected with *C. jejuni* receiving ciprofloxacin failed microbiologically, one also failed clinically. Although both pretreatment isolates were susceptible to ciprofloxacin, the post-treatment MICs were  $32 \mu\text{g ml}^{-1}$ . The study showed that mutation in *C. jejuni* could occur *in vivo* and result in clinically significant resistance.

Piddock (1995) cited several reports which documented that up to 20% of patients relapsed after treatment for enteritis due to the emergence of a resistant isolate with ciprofloxacin MICs of  $32 \text{ mg l}^{-1}$  or higher. This statement by Piddock (1995) is somewhat misleading and close reading of the respective references presents a rather different story.

In the study of Segreti *et al.* (1992), it is pertinent to ask why was clinical failure evident in only one of the two cases considering both infecting strains had post-treatment MICs of  $32 \mu\text{g ml}^{-1}$ . Assuming that the isolated strains were indeed those causing infection one needs to consider the concentration of ciprofloxacin to which the infecting strains are exposed. Serum levels of ciprofloxacin were available for both patients 2 h after oral administration and were equivalent to  $1.3$  and  $8.4 \mu\text{g ml}^{-1}$  for patients 1 and 2 respectively. Stool levels were only available for patient 2 and were equivalent to  $360 \mu\text{g ml}^{-1}$ . Although the paper doesn't appear to identify which of the patients exhibited clinical cure it would be interesting to speculate that it was patient 2 with a presumably higher level of ciprofloxacin in the intestinal tract. Indeed it is the ratio of available concentration of ciprofloxacin to MIC that is the determining factor for clinical outcome and thus, as elegantly described by Drusano *et al.* (1998) the MIC of the organism being treated is of secondary importance. Drusano *et al.* (1998) considered a study with 134

patients with a microbiologically documented infection and MICs determined for levofloxacin. Logistic regression analysis was performed for clinical and microbiological outcome employing pharmacodynamic covariance as well as other demographic and clinical covariates. The result was clear-cut with the peak concentration to MIC ratio being significantly linked to both clinical and microbiological outcome. The authors further analysed the data and found that a peak/MIC ratio of >10 was associated with both the best clinical and microbiological outcomes. These findings are consistent with the work of Forrest *et al.* (1993) and agree with the data of Blaser *et al.* (1987). In an earlier neutropenic rat model study Drusano *et al.* (1993) had shown that outcome for treatment of serious infections by the fluoroquinolone lomefloxacin was significantly better when the peak concentration/MIC ratio was 20:1. The authors used wild type and resistant mutants of *Pseudomonas aeruginosa* in this study and explained the improvements in outcome as being due to the suppression of the more resistant mutants in the population at the elevated peak concentration/MIC ratio.

#### Resistance development is not in doubt but .....

It is without dispute that microbiological resistance in *Campylobacter* spp. occurs as a result of mutation in the *gyrA* gene with single point mutations most frequently causing a four- to eightfold shift in the MIC. What is clear is that if a high enough peak concentration to MIC ratio can be achieved not only may the parent organism be killed but also the 'resistant' mutant. This no doubt goes some way to explaining the nature of clinical cure for 'resistant' isolates and explains why in the study of Segreti *et al.* (1992) one of the patients showed clinical cure as the ratio of peak concentration to MIC did exceed 10. Although not presenting any data for *Campylobacter* spp. MacGowan *et al.* (2000) reviewed the use of *in vitro* pharmacodynamic models of infection with regard to optimization of fluoroquinolone dosing regimens. These workers concluded that there is a consensus that high  $C_{max}/MIC$  values are important in preventing emergence of resistance and that whereas most data suggest that AUC/MIC is the best predictor of antibacterial effect, larger less frequent doses may be more effective than smaller more frequent doses.

Indeed typical faecal concentrations following oral dosage of ciprofloxacin suggest that *Campylobacter* spp. with MICs of  $32 \mu\text{g ml}^{-1}$  should in many cases be clinically susceptible. Brumfitt *et al.* (1984) in a study with 12 healthy male subjects aged 19–40 years showed ciprofloxacin concentrations in the faeces immediately post-treatment ranging from 185 to  $2220 \mu\text{g g}^{-1}$  following a 500 mg oral dose twice a day. Only two of the subjects had a concentration less than  $300 \mu\text{g g}^{-1}$ .

Robinson *et al.* (1985) measured ciprofloxacin in the faeces following the treatment of acute traveller's diarrhoea and demonstrated that the mean faecal levels exceeded  $500 \mu\text{g g}^{-1}$  during days 4 and 5 of therapy and for up to two days after therapy. In a study with marmosets Goodman *et al.* (1986) demonstrated that ciprofloxacin was able to clear *Campylobacter* spp. from chronically infected animals. Stool concentrations showed a mean concentration of  $49.2 \mu\text{g g}^{-1}$ , which commonly exceeded the MICs by 100-fold.

Cofsky *et al.* (1984) showed peak drug concentrations of norfloxacin in the faeces to range from 207 to  $2716 \mu\text{g g}^{-1}$  following a single 400 mg dose to 12 healthy volunteers.

Brismar *et al.* (1990) measured ciprofloxacin levels in a study on the effect of faecal flora in 21 patients undergoing elective colorectal surgery. The patients received two oral doses of 750 mg ciprofloxacin 12-hourly before surgery, 400 mg intravenously at the induction of anaesthesia and 400 mg again 12 h later. Faecal concentrations were in the range  $<0.1$ – $858 \mu\text{g g}^{-1}$ . In reviewing faecal concentrations of fluoroquinolones Korten and Murray (1993) considered that the widely varying figures quoted in the literature are possibly because of different study methods and distinct patient characteristics. Unfortunately we do not have data which relates area under the concentration-time curve for ciprofloxacin in the gastro-intestinal tract and we thus have to consider single value concentrations although in most cases these can be assumed to be 'steady-state' concentrations. Thomas *et al.* (1998), however, in a study evaluating factors associated with the development of bacterial resistance in acutely ill patients observed that a  $C_{max}/MIC$  ratio of approximately 5:1 may correspond to an  $AUC_{0-24}/MIC$  ratio of 100 for ciprofloxacin, whether this holds for the gastro-intestinal tract remains to be seen. What we do know is that all fluoroquinolones are found in much higher concentrations in the gastrointestinal tract than in serum (Korten and Murray, 1993). Indeed Krueger *et al.* (1999) established that mean ciprofloxacin concentrations in the faeces of  $185.3 \pm 158.7(\text{SD}) \mu\text{g g}^{-1}$  can be achieved in hospitalized patients following intravenous administration of 200 mg ciprofloxacin bid. Assuming the relationship between the ratios to be true it is interesting to consider the recent study from Bakker-Woudenberg *et al.* (2002) who support the view that it is the area under the concentration time curve and the peak drug concentration that is of primary importance for successful clinical outcome. These workers point out that most treatment failures with ciprofloxacin are a consequence of high MIC, low AUC, or both and that a peak drug concentration/MIC ratio of 10 or 20 has been shown both *in vitro* and *in vivo* to prevent the emergence of resistant mutants during fluoroquinolone therapy. They acknowledged the difficulty in achieving AUC/MIC

ratios of 125–250 in serious infections caused by pathogens such as *Staphylococcus* and *Pseudomonas*, species that are only marginally susceptible to fluoroquinolones on the basis of MICs. Considering the above and the concentrations of ciprofloxacin achieved in the gastrointestinal tract it is not surprising that clinical cure can be demonstrated for organisms with an MIC of 32 µg ml<sup>-1</sup>.

There is clearly data to show that clinical resistance is not a consequential function of microbiological resistance as determined by the currently used arbitrarily set breakpoints.

#### Are there other non-antimicrobial factors implicated in resistance development?

A final point regarding interpretation of data must take into account the recently published paper of Sánchez *et al.* (2002) who showed that *Campylobacter* isolates from immersion-chilled broilers had a higher incidence of resistance to nalidixic acid and related fluoroquinolones than isolates from air-chilled broilers. This was not true for all antibiotics as the tetracycline resistance frequency was higher in air-chilled than immersion-chilled broilers. It was clear that the chilling method used during processing influences the microbial profile of post-chilled broilers. What was particularly interesting in this study is that enrofloxacin susceptibility was also determined using E-test and very few isolates were determined as resistant to enrofloxacin, 8.3% from immersion chilling and 0% from air-chilling compared with 58.3% ciprofloxacin resistance from immersion chilling and 18.2% from air-chilling. The authors speculated on the effects of the different chilling regimes. Immersion chilled birds came from a wider range of farms in Missouri, whereas air chilled birds were less diversely distributed. In addition the heterogeneity of the strains present in the water tank may have created a larger gene pool from which contamination could have arisen. Whereas low levels of antimicrobial agents, chlorine and organic acids may reduce the numbers of susceptible organisms, allowing the selection of resistant strains (Archer, 1996), these stressing agents may act to activate mutational loci thus increasing resistance to these agents (Archer, 1996; Alekshun and Levy, 1997). As an example, the *mar* operon present in *E. coli* and other pathogens provides increased resistance to antimicrobial agents including fluoroquinolones and oxidative stress agents including chlorine, sanitizers and some organic acids (Alekshun and Levy, 1997). It might be expected that the same would be true for the *cmeABC* pump recently described in *Campylobacter jejuni* (Lin *et al.*, 2002). It is thus clear that there are factors other than the use of antibiotics within the production phase of poultry production that will contribute to the emergence of antibiotic resistant strains. Indeed Miché and Balandreau (2001)

demonstrated that hypochlorite, routinely added to drinking water in poultry houses and used in chiller tanks, was responsible for an increase in the frequency of nalidixic acid-resistant mutants of *Burkholderia vietnamiensis*. The mutation was a single amino acid substitution in the *gyrA* gene.

#### Conclusions

It is thus clear that the simple question, 'resistant or sensitive' can be extremely misleading when considering *Campylobacter* spp. There are many factors which contribute to the generation of what is an often and clearly ill used descriptor. A better understanding of the factors that influence the generation and use of this parameter will surely lead to more rational debate when considering fluoroquinolone use and *Campylobacter* spp. As multiple techniques are now available for detection of *Campylobacter* spp. from a range of samples we must consider use of such techniques as part of routine procedure. We can with confidence draw a number of conclusions from the literature and we need to be mindful of such conclusions when interpreting *Campylobacter* data.

- (a) The diversity of *Campylobacter* spp. in samples from poultry and from man is far greater than that initially considered to be the case.
- (b) Isolation methods from poultry usually involve enrichment techniques which will favour the isolation of the faster growing strains.
- (c) Isolation procedures will tend to overestimate the numbers of *C. jejuni*, particularly from human and companion animal cases, as strains such as *C. upsaliensis* tend to be missed because of their sensitivity to the antimicrobials used in culture medium. *C. upsaliensis* has been shown to be present in more than 10% of cases of human infection.
- (d) Sampling bias suggests that the tested populations of *Campylobacter* spp. are not representative of the sources from which they were sampled be they poultry, man or the environment.
- (e) Samples whether they are from poultry or man are commonly known to support multiple species of *Campylobacter* and indeed multiple strains of the same species. It is therefore not always possible to isolate a single strain from a human faecal sample and attribute it as the disease causing strain.
- (f) It is crucial that improved and standardized methodologies be utilised to clarify the role of campylobacters in human disease and that consensus is reached with regard to working definitions of resistant and susceptible. In order to achieve these objectives there must be greater collaboration between the laboratory and the clinician.

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