Methodology article

**Phenotyping of Campylobacter jejuni and Campylobacter coli by a quantitative antibiogram [MIC] typing scheme using Euclidean distances [QATED]**

John E Moore* and Colin E Goldsmith

Address: Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast BT9 9PAD, Northern Ireland, United Kingdom

E-mail: John E Moore* - jemoore@niphl.dinet.co.uk; Colin E Goldsmith - colingoldsmith@hotmail.com

*Corresponding author

Abstract

**Background:** Enteropathogenic *Campylobacter jejuni* and *C. coli* are presently the most common cause of acute bacterial gastroenteritis in the developed world. An understanding of sources and means of transmission of *Campylobacter* is an essential factor in order to reduce the incidence of *Campylobacter*-related gastroenteritis in man. Consequently a reproducible, sensitive and well-standardised typing scheme is critical in the successful discrimination of strains and in the subsequent investigations of outbreaks. For this purpose, a phenotypic typing scheme based on quantitative antibiogram determination based on Euclidean distance (QATED), was developed.

**Results and Conclusion:** The results obtained with this typing scheme demonstrated that individual livers of colonized pigs could be infected with multiple strains of *Campylobacter* spp. and subspecies types. In conclusion, phenotyping of *Campylobacter jejuni* and *C. coli* by QATED is a simple, inexpensive and discriminatory sub-species characterisation scheme, which may be useful in primary diagnostic clinical laboratories, where no specialist *Campylobacter* phenotyping or molecular genotyping schemes exist. It is especially suitable for food-borne outbreak investigations in the community, where a rapid and local response is required to aid with public health epidemiological investigations.

Introduction

The understanding of sources and means of transmission of Campylobacter is an essential factor in order to reduce the incidence of Campylobacter-related gastroenteritis in man [1]. Presently, there is growing concern over the emergence of antibiotic resistance in campylobacters. Although oral antibiotics are not normally prescribed in the majority of cases as the disease is usually self-limiting, antimicrobial therapeutic intervention may be required due to the immunocompromised state of the patient or any other predisposing complication. Furthermore, there is growing concern regarding rates of fluoroquinolone resistance in human isolates and the associated employment of similar agents within animal production [2]. Consequently, there is a need to develop typing schemes that will address the specific issues of increasing antibiotic resistance, mainly by examining extended antibiograms, which may be useful in patient management, as well as in epidemiological surveillance of evolving resistance patterns, as current surveillance
programmes are often limited in the quantity of data that they are able to produce.

Consequently a reproducible, sensitive and well-standardised typing scheme is important in the successful discrimination of antibiotic resistance patterns of strains in the investigation of outbreaks. Hence in order to elucidate the sources and modes of transmission of antibiotic resistance characteristics, it is essential to use epidemiological typing methods which discriminate between different strains but which are reliable and reproducible in the recognition of similar strains. In addition, employment of molecular-based typing schemes, although highly discriminatory, are not available in most primary diagnostic laboratories and hence necessitates the use of widely available phenotypic techniques and reagents.

The objective of this study was to develop a simple phenotypic characterisation scheme for *C. jejuni* and *C. coli*, based on a quantitative comparison of MIC antibiograms using Euclidean distances [QATED].

**Results**

Porcine *C. jejuni* and *C. coli* isolates examined showed a wide variation in their resistances to the antimicrobial agents tested. In general, the *C. coli* strains were more resistant than the *C. jejuni* strains and there was a greater resistance range with the veterinary antimicrobials than with the clinical antibiotics. All 30 isolates were susceptible to the antimicrobial agents employed with the exception of monensin sodium, which did not inhibit any isolate of either *C. jejuni* or *C. coli* at the maximum concentration tested (500 µg ml⁻¹). Specific antibiotic resistance to the antibiotics tested have been published previously [3]. Resistance phenotypes were shown to be stable on repeated sub-culture.

MIC values were noted and an arbitrary value ranging from 1 to 14 was assigned corresponding to the dilution at which the MIC occurred, where 1 represented zero inhibition of growth and 14 represented an MIC of 500 µg ml⁻¹. Each isolate was characterised by its combination of its resistance pattern over the 11 antimicrobial agents examined and distinctive profiles, corresponding to unique resistance phenotypes were designated antibiogram types (ATs). An AT pattern was created for each isolate and these patterns were compared for similarity by statistical analyses. No two patterns were identical either for *C. jejuni* or *C. coli* showing the diverse range of resistances within the Campylobacter isolates examined. Pairwise comparisons between ATs were analysed statistically by calculating the Euclidean distance ([1-(x-xi)/range]) between clustered pairs of strains, which gave an unweighted matrix of coefficients of related distance over the 11 antimicrobials examined. From this matrix of coefficients of phenotypic distance, a dendogram (Fig. 1) was generated as a model for the phenotypic relatedness among the 30 ATs examined, employing a Genstat 5 (version 2.2) statistical package in conjunction with a VAX/VMS5 computer system [4].

**Discussion**

Enteropathogenic *Campylobacter jejuni* and *C. coli* are presently the most common cause of acute bacterial gastrointestinal infections in the developed world, as well as an important gastrointestinal pathogen in developing and underdeveloped countries [5]. The majority of human cases (99%) present as sporadic in nature, unlike other gastrointestinal infections, such as *Salmonella typhimurium*, which are generally more related to large institutional outbreaks. However it is unclear whether or not the epidemiology of human *Campylobacter* infections are related due to poorly developed and evaluated sub-species typing schemes for this organism, as well as a subsequent lack of commitment for national surveillance programmes in developed nations, due to a perceived caution as to the value of such data, originating from such typing schemes. As a result of this, coupled with increasing diagnostic laboratory financial constraints, many diagnostic laboratories will only normally confirm antibiotic sensitivity profiles, to aid with the management of the infected patient.

Phenotyping and molecular genotyping expertise is normally not present in most primary diagnostic clinical laboratories, due to the relative complexity and high-costs associated with having such facilities available locally. Consequently small local outbreaks due to this organism may not be detected, where a rapid and local response for the clinical microbiology laboratory is essential in order to aid with public health epidemiological investigations and containment of contaminated foodstuffs or water. Presently, with a lack of phenotypic and genotypic schemes available locally, sub-species strain relatedness is crudely based on a number of qualitative phenotypic parameters, such as differences in colonial morphology and on limited antibiogram typing based on a qualitative comparison of a limited number of disc diffusion antibiotic susceptibility assays.

The cost and applicability of this scheme is compared to another phenotyping scheme and a genotyping scheme (PCR-RFLP) employing the flaA system and is shown in Table 1. Overall, although we found the technique to be reproducible, it is important to employ rigorous quality controls and laboratory standards. These should include
standardisation of media preparation, within a single production batch/lot number and employment of reference and internal strains of known antimicrobial susceptibility, which should be employed on each set of susceptibility assays performed.

The QATED typing scheme allowed for the computation of a mathematical index of relatedness between the 30 isolates examined. This scheme offers several advantages as a first-line sub-species strain characterisation method, particularly for outbreak confirmation in local primary diagnostic laboratories, as (i) most laboratories will be familiar and experienced with antibiotic susceptibility [MIC] testing protocols, (ii) the method may be made more rapid by the employment of E-test MIC strips, (iii) all isolates are typeable, (iv) there is a high degree of inter-laboratory reproducibility and intra-laboratory repeatability with this method, (v) this method will demonstrate mathematical and quantitative comparison data with regard to relatedness of strains – an attribute which is not achievable by either bio-, sero- or phage-typing protocols, as these tend to be absolute in nature, i.e. either totally similar or different.

Although campylobacteriosis is a self-limiting infection, a minority of patients requires treatment. Antibiogram determination as a standard procedure in routine laboratories would increase effectiveness of treatment. It would also be an important tool to monitor increase of resistance against agriculturally applied antibiotics. Since resistance to certain antibiotics is rapidly rising in C. jejuni, this issue is highly relevant at present.

In conclusion, phenotyping of Campylobacter jejuni and C. coli by QATED is a simple, inexpensive and discriminatory sub-species characterisation scheme, which may be useful in primary diagnostic clinical laboratories, where no specialist Campylobacter phenotyping or molecular genotyping schemes exist, especially for food-borne outbreak investigations in the community, yet where a rapid and local response is required to aid with public health epidemiological investigations.

Table 1: Comparison of suitability of QATED, PCR-RFLP and serotyping for employment of outbreak analysis in the routine diagnostic laboratory

| Laboratory parameter          | QATED† | PCR-RFLP* | Serotyping |
|------------------------------|--------|-----------|------------|
| Time to result               | 24 h   | 9 h       | 2 h        |
| Relative cost/isolate        | moderate | high     | high       |
| Ease of use                  | simple | complex  | average    |
| Repetitability               | good   | good     | good       |
| Requirements for complex equipment | average | high    | average    |
| Applicability to routine diagnostic laboratory | good | poor | poor† |

†QATED, quantitative antibiogram typing based on Euclidean distance.
* RFLP, restriction fragment length polymorphism; †Based on difficulty in obtaining quality antisera

Materials and methods

Origin of Campylobacter spp
Campylobacter spp. were isolated from pork livers (n = 400) from bacon pigs (n = 37 herds) obtained at six pork processing plants in N. Ireland. Deep tissue areas were sampled immediately post-evisceration on Campylobacter selective agara and revealed that 6% of livers were infected with Campylobacter spp., consisting of C. coli (67%), C. jejuni (30%) and C. lari (3%).
Preparation of bacterial isolates
A total of 30 isolates comprising of 10 isolates of *C. jejuni* and 20 isolates of *C. coli*, were included in this study. Each isolate was passaged twice on non-selective basal medium without the addition of antibiotic agents, i.e. blood agar no.2 (Oxoid Ltd., Hampshire, England) supplemented with defibrinated horse blood (Oxoid Ltd) to obtain non-stressed organisms for in vitro antimicrobial susceptibility testing.

Antimicrobial susceptibility testing
Minimum inhibitory concentration (MIC) determinations were carried out by the agar incorporation method. Late log phase cultures (18 h) were prepared by culturing cells on blood agar no.2 (Oxoid Ltd., Hampshire, England) supplemented with defibrinated horse for 18 h (overnight) at 37°C in microaerophilic conditions (5% O₂ [V/V], 10%CO₂ [V/V] & 85% [V/V] N₂). Cells were harvested from duplicate plates and were resuspended in 0.1% [w/v] peptone saline solution. Cells were inoculated onto the surface of Mueller-Hinton agar using a multipoint inoculator (Denley Instruments, England). A 3 x 3 needle arrangement was adopted in order to avoid cross-contamination of adjacent strains. Each needle delivered approximately log10 6.70 cfu on to the surface of the agar surface. The medium used for susceptibility testing was unsupplemented Mueller-Hinton agar except for cotrimethoxazole where this medium was supplemented with 5% defibrinated sheep blood. Mueller-Hinton agar was chosen as it is nutritionally richer than other antibiotic susceptibility test agars, such as Iso-Sensitest agar (Oxoid CM471) and has been used widely in other *Campylobacter* susceptibility studies [6,7]. Plates were poured and incubated in the dark at 30°C for 24 h, in order to dry the surface of the medium to encourage rapid absorbance of the liquid and thus avoid surface smear between adjacent strains. In addition, the agar concentration of the medium was increased to 2% [w/v] with Agar no. 1 (Oxoid) in all assays in order to avoid swarming of isolates across the agar surface. All antimicrobial agents were made up immediately prior to incorporation into the Mueller-Hinton agar to give 14 binary concentrations of the medium was increased to 2% [w/v] to obtain non-stressed organisms for in vitro antimicrobial susceptibility testing.

(i). clinical: cephalosporin C, ciprofloxacin hydrochloride, cotrimethoxazole (20:1 sulphamethoxazole:trimethoprim), erythromycin, gentamycin,
(ii). veterinary: dimetridazole, monensin sodium, sulphadimidine, sulphamethoxazole, tetracycline,
(iii). food: nisin.

Acknowledgements
JEM was supported in part by a Department of Agriculture for Northern Ireland Postgraduate studentship. This work was supported by the Department of Health & Social Services (Northern Ireland).

References
1. Engberg J, Aarestrup FM, Taylor DE, Gerner-Smidt P, Nachamkin I: Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: Resistance mechanisms and trends in human isolates. *Emerging Infectious Diseases* 2001, 7:24-34
2. Moore JE, Crowe M, Heaney N, Crothers E: Antibiotic resistance in *Campylobacter* spp. Isolated from human faeces (1980–2000) and foods (1997–2000) in Northern Ireland: an update. *Journal of Antimicrobial Chemotherapy*, 2001
3. Moore JE, Madden RH, Kerr JR, Wilson TS, Murphy PG. Erythromycin-resistant thermophilic *Campylobacter* species isolated from pigs. *Veterinary Record* 1996, 138:306-307
4. Lawes Agricultural Trust: *Genstat V*. Release 1.3. *Rothamsted Experimental Station*, England. 1990
5. Skirrow MB, Benjamin J: *Differentiation of enteropathogenic* *Campylobacter*. *Journal of Clinical Pathology* 1980, 33:122
6. Butzler JP, Dekeyser P, Lafontaine T: *Susceptibility of related vibrios and Vibrio fetus to twelve antibiotics*. *Antimicrobial Agents and Chemotherapy* 1974, 5:86-89
7. Anon: *US National Committee for Clinical Laboratory Standards Performance standards for antimicrobial susceptibility testing. Second information supplement.* NCCLS document M100-S2. Villanova, Pennsylvania, U.S.A. 1987