Methodology article

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

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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-bome 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 or greater. 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 ([l-(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 gastroenteritis 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 subspecies 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 the presence of this organism to the genus level. In addition, it is clinically important to have an identification of this organism to at least the genus level, as well as 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

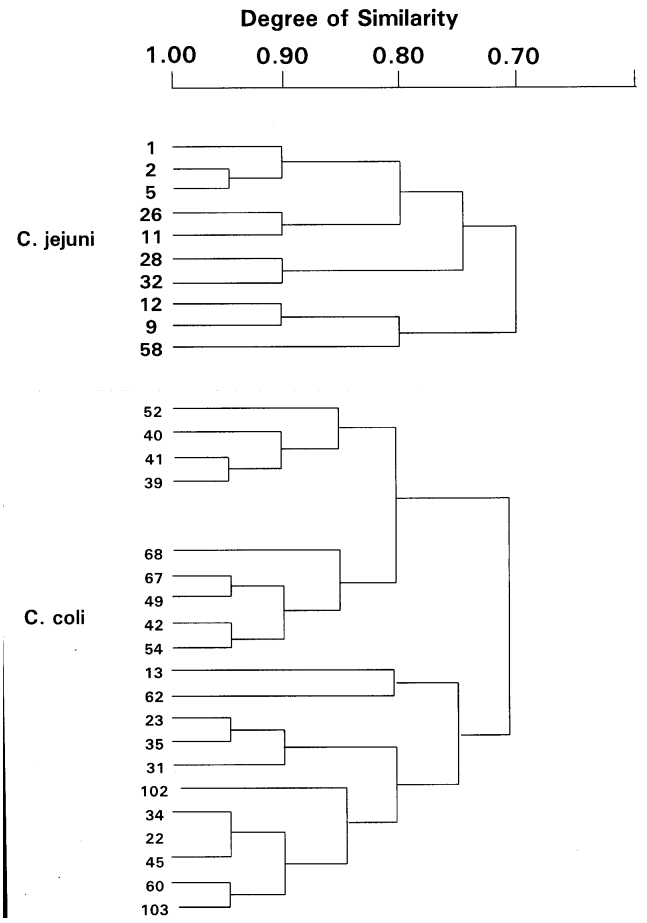

Figure 1
A dendrogram showing relatedness of 10 isolates of *C. jejuni* and 20 isolates of *C. coli* based on QATED typing.

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 foodborne 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
Repeatability	good	good	good
Requirements for complex equipment	average	high	average
Applicability to routine diagnostic laboratory	good	poor	poor[1]

†QATED, quantitative antibiogram typing based on Euclidean distance,
* RFLP, restriction fragment length polymorphism; 1. 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% O_2 [V/V], 10%CO_2 [v/v] & 85% [v/v] N_2). 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 3×3 needle arrangement was adopted in order to avoid cross-contamination of adjacent strains. Each needle delivered approximately $\log_{10}$ 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 choosen 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 dilutions (0–500 μg ml^{-1}). Antibiotic-free plates were inoculated as controls. After inoculation, plates were incubated at 37°C for 24 h prior to examination. Complete or partial confluence was judged to be resistant, whilst no growth was judged to be sensitive for all combinations of each antibiotic and concentration. The MIC value was defined as the lowest concentration of antibiotic which gave a complete absence of growth following 24 h incubation.

Antmicrobial agents tested

The following antimicrobial agents were tested:-

(i). clinical: cephalosporin C, ciprofloxacin hydrochloride, cotrimethoxazole (20:1 sulphamethoxazole:trimethoprim), erythromycin, gentamycin,

(ii). veterinary: dimetridazole, monensin sodium, sulphadimidine, sulphamethoxazole, tetracycline,

(iii). food: nisin.

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