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Isolation, identification and subtyping of *Campylobacter*: Where to from here?

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ABSTRACT

Campylobacter species are widely regarded as the most frequent bacterial cause of gastroenteritis in humans worldwide. Their main transmission routes are via contaminated food and water. For interventions to be effective, methods for the detection, identification and epidemiological subtyping must be sensitive, accurate and rapid. As yet, methods are not perfect, although several significant advances have been made in these areas in recent years. This paper provides a brief review and commentary on the current state of the art in the hope that it will help provide context for others in selecting, improving or developing these vital tools for research and diagnoses.

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1. Introduction

The seminal works of Butzler et al. (1973) and Skirrow (1977) demonstrated the considerable significance of *Campylobacter jejuni* and *Campylobacter coli* as causes of acute gastrointestinal disease and initiated a monumental number of research studies that has lasted over 35 years and continue to this day. These collected works have transformed our understanding of these organisms in almost every aspect of their study: taxonomy, genetics, evolution, epidemiology, pathogenicity, survival and beyond. Yet our knowledge of these clinically and economically important bacteria begins with some basic needs: without effective isolation, identification and subtyping procedures, diagnoses and interventions cannot begin, leaving additional studies struggling to find validity.

This is not intended to represent an authoritative review of developments in these areas. Instead, this paper aims to assess current needs and gaps in the field and perhaps provoke or even inspire others who wish to pursue a particular area.

2. Isolation methods

Isolation methods for the classic pathogens *C. jejuni* and *C. coli* from clinical samples are arguably as effective as they will ever become, with some caveats. The key characteristics of these organisms allowing growth and selection are understood, and the higher number of cells in a clinical (or animal faecal) sample make their recovery reasonably straightforward. The widespread use of growth temperatures of 42 °C to exploit the thermotolerant nature of *C. jejuni* and *C. coli* both promotes their growth and represses other bacterial species. In addition, antibiotic cocktails in selective media have become refined in their combinations and concentrations over time and have seen for good recovery rates. For food and water samples, and perhaps clinical samples requiring lengthy periods of transportation before analysis, the recovery of stressed cells can be challenging. Procedures using staged periodic increases in temperature to aid adaptation and recovery can be time consuming and overly complex (Corry et al., 1995). The use of immunomagnetic separation methods to concentrate all cells as used for certain *Escherichia coli* serotypes appears problematic, with the few studies undertaken on campylobacters showing limited efficacy when applied to naturally contaminated samples (Miller and Mandrell, 2005). The difficulties encountered thus far may perhaps be due to the antigenic diversity and variation of *C. jejuni* and *C. coli*, making it difficult for a single antibody (as used to coat the magnetic beads that form the basis of the assay) to recognise and thus capture all strains. For water samples, methods such as pre-filtration to concentrate cell densities followed by pre-enrichment and culture are used; the subsequent use of a 0.65-μM membrane filter has been found to improve the specificity of the method (Jokinen et al., 2012). In any case, exposure to environmental stresses of various forms – temperature, pH, A_w, starvation – triggers a response that often results in the so-called "viable but non-culturable" (VBNC) form that appears to be one capable of surviving as an intact and potentially infectious

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agent yet resistant to conventional culture (Jackson et al., 2009). There is strain-to-strain variation in the responses and degrees of survivability (On et al., 2006; Jackson et al., 2009) and documented changes in certain pathways and expression profiles when strains are subjected to different stresses (Stintzi and Whitworth, 2003). Nonetheless, our understanding of the underlying processes resulting in VBNC formation and certainly of the processes that lead to their recovery from this dormant state (shown to be possible when introduced to live animals) (Jackson et al., 2009) is far from complete. Additional studies on the survival mechanisms of *Campylobacter* would perhaps yield new ideas for improvement of the recovery of environmentally stressed campylobacters from important vectors including food and water.

Considerably less studied are the prevalence and importance of species other than *C. jejuni* and *C. coli*, especially as related to food as a source of illness. We do know there is an extensive range of related taxa and that many *Campylobacter*, *Arcobacter* and *Helicobacter* species are known or suspected pathogens (On, 1996; Man, 2011). Table 1 summarises the taxonomic status and clinical associations of *Campylobacter* species at present. Most of these bacteria have different growth requirements to *C. jejuni* and *C. coli*, and until recently, specific methods for isolation have not been applied. It is important to realise that even for relatively closely related pathogens such as *Campylobacter upsaliensis*, the presence of selective antibiotics in commonly used media significantly hampers recovery (Acke et al., 2009). Possibly even further neglected is the requirement for gaseous hydrogen in the cultivation atmosphere, without which many species cannot grow (Vandamme et al., 2005). From that perspective, the lack of commercially available "gas packs" to generate a microaerobic atmosphere that includes H_2 is unfortunate, especially since the growth of *C. jejuni* and *C. coli* are also enhanced. What is, however, notable is that a method designed with the objective in mind of recovering multiple Campylobacteraceae (Lynch et al., 2010) is proving instrumental in isolating a wide range of these taxa from foods (Lynch et al., 2011). Most notable perhaps is *Campylobacter concisus*, which has been found to equal *C. jejuni* and *C. coli* frequencies in human diarrhoeal samples in many specific studies (Engberg et al., 2000; Man, 2011). Although both the taxonomy (Aabenhus et al., 2005) and clinical importance of *C. concisus* require further study to substantiate its role in human disease, its frequency in human illness (Engberg et al., 2000; Man, 2011), presence in foods and domestic pets (Petersen et al., 2007; Lynch et al., 2011) and possession of pathogenic traits (Kaakoush et al., 2011) urges additional focus. It may well be a major explanation for the 70% of gastroenteritis cases that, each year, go without diagnosis.

3. Identification methods

Successful isolation of a strain logically leads to the next stage—the identification of the isolate, requiring that its characteristics, phenotypic, genotypic or both, however determined, match sufficiently with those of a given, known species to positively assign it. The matching data can range from biochemical test results in a simple table or primer sequences in a PCR reaction. Identification strategies therefore range from comparative ("What is it?") to deterministic ("Is it this?"). The problems in the identification of *Campylobacter* and phylogenetically related species including *Arcobacter* and *Helicobacter* are well established (On, 2005) and include their biochemically rather inert nature and their complex taxonomy. The wider phylogenetic group, Epsilonproteobacteria, contains over 100 taxa (listed online at www.bacterio.cict.fr/classifordersclasses.html#Epsilonproteobacteria). While several genera comprise only free-living species unlikely to cause illness in humans or animals, *Arcobacter* and *Helicobacter* species may occur in the same hosts and cause diseases similar to those associated with *Campylobacter*. In general, a tendency to grow preferentially at temperatures between 18 °C and 25 °C and in aerobic conditions are useful in classifying *Campylobacter*-like strains as *Arcobacter*; no overarching phenotypic feature that is easily determined in a routine laboratory can be used to readily identify *Helicobacter* spp. in the same way.

The rapid and constant evolution of the taxonomy of the Epsilonproteobacteria also presents a major challenge. At least one new taxon – subspecies or species – has been validly described and added to the group since 1988. This does not include many taxa that are published in journals other than the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM: the only journal in which taxonomic descriptions are formally recognised by the international community), which may not necessarily have been delineated by appropriate methods (e.g. "*Helicobacter* spp. *Mainz*," shown to be *H. cinaedi*) (Vandamme et al., 2000). On occasion, the taxonomic status of even validly described taxa is revised (e.g. *Campylobacter hyoilei*, shown to be *C. coli*) (Vandamme et al., 1997). Although these changes can prove challenging for non-specialists to keep updated, the excellent, free and frequently updated online resource authored by J. P. Euzéby mentioned above provides clear guidance on the current status of internationally accepted bacterial nomenclature (http://www.bacterio.cict.fr/). This, however, only facilitates the refreshment of one's knowledge base to be able to recognise new taxa that may be involved with clinical or other environments under investigation. Current taxonomic rules demand that new taxa be described with sufficient phenotypic traits that allow for ready diagnosis in a routine setting, but some individual tests may still be relatively specialised. The challenges of accurate identification using conventional phenotyping certainly drove developments in the use of molecular methods in particular, and PCR has become a widely used diagnostic tool in routine laboratories. Consequently, an array of assays aimed at accurately identifying individual taxa has been developed based on genomic data. The inherent trust in an assay described in the literature and based on DNA sequences can be misplaced. Initially, the accuracy of the method will depend not only on the quality of the design but also on the quality of the validation, which needs to take into account infraspecific (i.e., within species) as well as intraspecific (i.e., between species) biological variation. Previous comparisons of existing tests have revealed deficiencies in claims of specificity and exclusivity on that basis (On and Jordan, 2003; Debruyne et al., 2008). However, even where a test has been demonstrated at the time to be accurate, one cannot rule out the possibility that species described *since* the assay description may be detected with a PCR designed for another, earlier described species. Indeed, work undertaken at the Institute of Environmental Science and Research (ESR) as part of the European Commission "MoniQA" project on the standardisation of tools for detecting food-borne hazards has shown that more recently described species including *Campylobacter lari* subsp. *concheus* and *Campylobacter volucris* do yield positive results in many PCR assays originally designed only for *C. jejuni* and *C. coli* (On et al., 2013). An algorithm is suggested for workers using such tests to revalidate their existing protocols and continually certify their accuracy (On et al., 2013). Clearly, there is a need for continued scrutiny and vigilance to assure accurate diagnoses for clinical and epidemiological purposes.

In contrast with highly specific methods such as PCR that confirm the identity of an isolate if a result is obtained but leave the laboratory no better informed if results are negative, comparative methods where data are evaluated against similar data for known taxa have the advantage of at least providing an indication of where the unknown organism is placed in taxonomic space. The portability and ease of use of 16S rRNA gene sequencing, and the continually growing and evolving repository of data in public databases such as GenBank, have made this approach probably the most widespread DNA-based method for prokaryotic identification. For Epsilonproteobacteria, however, the limitations of 16S rRNA gene sequences as an entirely appropriate means to delineate species are well known and include both examples where strains of a single species exhibit such diversity they may be classified as different taxa (*Campylobacter hyointestinalis, Helicobacter cinadei*) and distinct species with insufficient divergence

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