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The Consequences of Genome Instability and Clonality to Genotypic Identification of *Campylobacter jejuni**

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Introduction

Since the development of the first techniques to make a genetic fingerprint of bacterial isolates, over 20 years of putting genotyping procedures into practice has passed. Whereas these tools have helped identifying virulent strains or subspecies, emerging pathotypes, or routes of transmission for certain bacterial pathogens, the application of genotyping methods has disappointedly been of little use to understand the molecular epidemiology of *Campylobacter jejuni*. *C. jejuni* is a major cause of bacterial enteritis world-wide, and is a colonizer of many warm-blooded animals, including birds. All genotyping data suggest that a high degree of diversity exists between isolates. As will be discussed, the population is not completely clonal, as inter-strain recombinations seem to occur frequently, nor is it completely non-clonal. Genetic instability occurs to a degree and frequency that is detectable with genotyping methods. Now that the tools to differentiate isolates that are genetically divers are available to the community, it is frustrating to see how little the generated data have helped our general understanding of the epidemiology of this food-born pathogen. This treatise will discuss what the causes and consequences are for non-clonality and genetic instability.

Genotyping of a clonal bacterial population

Ideally, one bacterial clone should give rise to one genotype only. When genotyping methods have different degrees of discrimination, isolates belonging to one genotype as determined with a low-discriminatory method can be further differen-

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tiated with a second, higher discriminatory method. Taking such differences in discriminatory level into account, all genotyping data should correlate within a clonal bacterial population, as illustrated in the top of figure 1. Bacterial species with a low level of genetic diversity and a strong clonal structure give rise to genotypes that are easy to interpret. Under such conditions, strain differentiation and identification can be based on single genotyping methods, providing these methods have sufficient discriminatory power. For *C. jejuni*, genotyping is less simple. This is so because the population is partly non-clonal and because genetic instability occurs to some degree.

How to determine a non-clonal bacterial population: MLST

A non-clonal bacterial population results from frequent horizontal gene transfer. When chromosomal DNA fragments are exchanged by natural transformation, the genetic lineage within the offspring of a single cell is lost. Offspring is no longer genetically identical to it's ancestry. The degree of non-clonality differs between species (1). Some bacterial species are completely clonal, with few different clones presently exisiting, or even comprising one single clone, such as *Yersinia pestis* (2) (which may have undergone sufficient point-mutations to allow a degree of subtyping by genotyping) where as other bacterial species are completely non-clonal (such as *Helicobacter pylori*) (3). It may seem confusing that isolates within a clonal population can still be sub-classified with the help of genetic methodology. However, even within the clonal population of, for instance, *Salmonella typhimurium*, point mutations do occur and when these give rise to differences in genotype, they can be used to sub-classify isolates within the bacterial population. Despite such point mutations, the overall clonal structure is not destroyed. Such accumulative point mutations are determined by Multi-locus sequence typing (MLST) (4), where the target genes are preferentially house-keeping genes that are under no selective pressure, low mutating, and evenly distributed over the genome. MLST typing involves the PCR and sequence analysis of, typically, 5–8 of such genes or gene fragments. In a clonal population, the MLST typing data completely match: certain sequence alleles for each gene will end up in certain combinations only. In a non-clonal population, such house-keeping genes can be horizontally transferred from one genomic context to another, so that the "wrong" allele is found in different genetic contexts. With MLST, the degree to which such genetic linkage is lost can be quantified. Therefore, MLST is a powerful technique to determine the degree of clonality within a bacterial population. From the available data it can be concluded that *C. jejuni* is partly non-clonal, meaning that intra-specific recombinations occur frequently in part of the population (5, 6). These data are in agreement with the genotyping data that have been accumulated over the years (7).

Although MLST is a powerful technique to determine the clonal relation within a complex bacterial population, it may not be the best technique for standard genotyping purposes. Not only is the technique elaborate, it is also optimally designed to

determine long evolutionary relationships, thereby ignoring recent minor genetic changes in genes under strong selective pressure. Such genetic changes may, however, have major effects on phenotype, including virulence, and this is what epidemiologists are most interested in. For strain identification in epidemiological studies, classical MLST may not be suitable, since virulence genes are not included in the analysis because they may be under selective pressure. However the addition of highly variable genes under selective pressure (such as the short-variable region of flagellin (8) may improve the performance of the technique for these specific demands.

Genotyping and serotyping of *C. jejuni*

Subtyping of *C. jejuni* has developed in parallel to that of other organisms. At first, typing was performed on the phenotypic level, with serotyping as the most obvious example. Although serotyping of *Salmonella* and *E. coli* isolates has been highly successful in identifying virulent pathotypes, this is less evident for *C. jejuni*. This is partly because the population is not completely clonal, and partly because there is such a high degree of diversity. The number of serotypes currently known (>100 heat-labile serotypes, >60 heat-stable serotyes) (9, 10) is still not sufficient and up to 30% of isolates remain untypeable (11). When genotyping methods became in use, it soon became apparent that genotyping and serotyping data do not always match. Only when dealing with clonal serotypes will the results of serotyping and genotyping (and the results of different genotyping data) correlate, as illustrated in figure 1.

Despite frequently occurring intra-specific recombinations, stable clones of *C. jejuni* have been reported to occur. In a study from the UK, a stable O:6, H:6 strain could be isolated 19 years apart from different geographical areas (12). In a study from South Africa, a stable O:41 strain was isolated over a period of 15 years (13). Isolates from Guillain Barré Syndrome (GBS) patients frequently belong to the serotype O:19 (14) and these isolates are near to genetically identical. In all of these examples, isolates of the same serotype will group closely together by high-discriminatory techniques, such as AFLP (15), and will be indistinguishable by genotyping techniques with less discriminatory power. These are examples of stable clones within the *C. jejuni* population.

Other studies in which genotype and serotype were combined, largely failed to correlate these data (see 7 for a review of this topic). The same is observed when two genotyping data (for instance PFGE and fla-typing) are combined: identical PFGE isolates can have different fla-types and vice versa. The same combination of genotypes is not always found, and conserved combinations of genotypes become very infrequent when a third method is included. This demonstrates that a large part of the *C. jejuni* population is non-clonal.

Even the isolates that share the same serotype may not be members of one clone. The frequently isolated serotypes O:2 and O:4 are partly non-clonal and geneti-

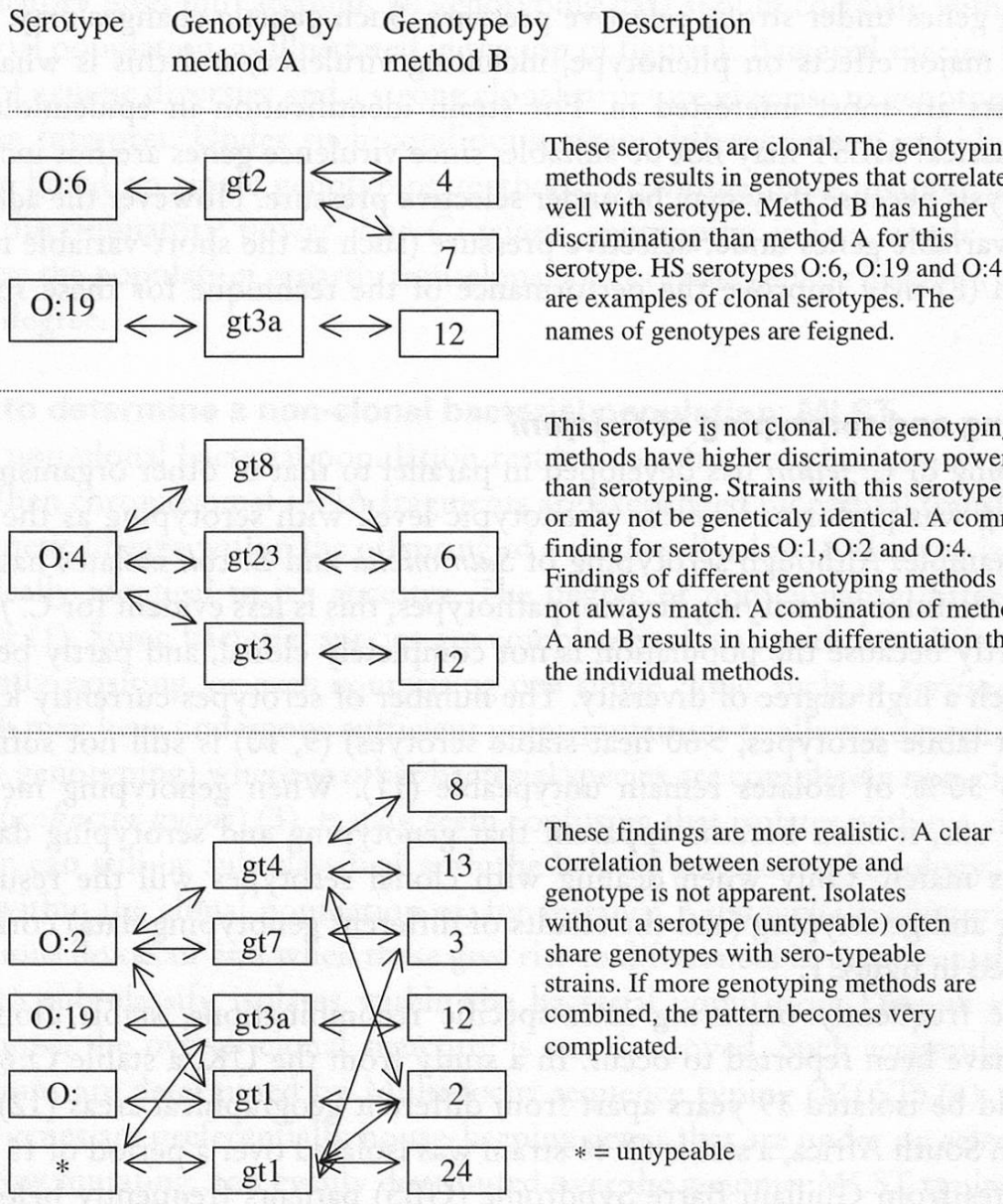

Figure 1 **Schematic of correlation between serotyping and genotyping methods in clonal, partially nonclonal, and non-clonal populations**

cally diverse, so that the genotyping data obtained with these isolates do not match. This also illustrates that serotyping is not the Golden Standard for *C. jejuni*. Several serotypes comprise of a group of isolates that have little more in common than their serotype, and serotyping can be regarded as a low-discriminatory technique for *C. jejuni*.

For clonal isolates, serotyping is as good as any other method, since all isolates are (near to) identical within one clone, thus the serotype will identify clones. This happened to be the case for O:19, and with it's coincidence with GBS, a revival of serotyping as a typing technique was predicted. However, the coincidence of a certain serotype with GBS may have been misleading. In South Africa GBS patients are

frequently infected with O:41 but not with O:19 (13) (the latter does occur in the region but has never been isolated from GBS patients). The *fla*-type of O:41 and O:19 is identical, and this reflects the genetic similarity of the two serotypes, which are more similar to each other than to any other serotype (as determined by AFLP) (13). The original observation of strain-association with GBS would had been equally apparent with *fla*-typing or any other typing method, since O:19 isolates are clonal. As could be expected, the *fla*-type occurring with O:19 and O:41 is now presented as a predicting marker for GBS (16). However, not every patient with an O:19 or an O:41 infection results in GBS, and GBS patients can have suffered a *C. jejuni* infection from a number of other serotypes (17, 18). This illustrates that other factors than the epitopes determined by serotyping are important in the onset of GBS. In fact, the coincidence with O:19 may have been partly misleading. The original observation that O:19 strains possess GM1-like antigens (19) led to the hypothesis that this antigen results in an auto-immune response in GBS patients. The fact that 30 % of *C. jejuni* isolates possess GM1-like epitopes (11) is ignored in this hypothesis.

Genotyping and genetic instability

Genotyping methods were introduced as an alternative to serotyping, with a number of advantages. Genotyping results in less non-typeable isolates (depending on the method), is generally available (again depending on the method), does not require specific antisera or other reagents, and is not influenced by phenotypic variation. The discovery that genetic variation could result in instability of genotype damaged this reputation. Genotypes can be unstable due to a number of mechanisms, which can be divided in those that require horizontal gene transfer (natural transformation) and those that do not require DNA transfer.

DNA transfer occurs in naturally competent species, including *C. jejuni* (19, 20) When isogenic mutants are grown together on a plate, within 72 hrs transformants arise at a frequency of 10^{-4} per CFU (21). However, gene transfer is much less common between strains than within one strain (as with isogenic mutants). It is unknown at which frequency DNA transfer occurs between strains under natural conditions.

Rearrangements within the genome can also occur. In this case transfer of DNA is not required. There are two types of recombinations that take place on a different size scale. Large size DNA fragments may recombine which can be detected by PFGE (22, 23) and possibly by ribotyping, but this would remain undetected with methods that zoom in on a single locus (or a number of small loci), such as *fla*-typing, multiplex PCR, MLST, or AFLP. Small size DNA fragments may also recombine, for instance in the case of gene duplication, and this has been shown to occur in the flagellin locus (21). Although in this experimental case an antibiotic resistance cassette was present in one of the two flagellin genes, which allowed easy

detection of the recombinants, it can be assumed that inter-flagellin recombinants occur under natural conditions also, which would affect *fla*-typing.

The frequency with which these phenomena occur under natural conditions is currently unknown. Their results may remain unnoticed when the genotypic method applied does not detect the change, or they may be misinterpreted for a change of strains, instead of a change of genotype within a strain. In a review article the observations that may be indicative of genetic instability were listed, with experimental suggestions to confirm or disprove bacterial lineage in such cases (24). When a single genotyping method is applied to strain identification, the occurrence of genetic instability is likely to be missed. More reliable data are obtained when two methods are combined, preferentially one that determines global genome types (such as PFGE or AFLP, possibly RAPD) and one that determines a single locus (such as *fla*-typing or serotyping).

The time scale of events leading to genetic instability and non-clonality must be taken into account. When a bacterial population is examined, the accumulative effects of ten-thousands of years can be detected. Non-clonality as a result of infrequent DNA transfer may be detected in a random population. However, if one population is studied in real-time (for instance during colonization of a single chicken flock), the time span may be too short to observe any instability or horizontal gene transfer. Similarly, most isolates display stability of genotype when cultured *in vitro*, even for "long term" cultures. Thus, for short-term epidemiological studies, horizontal gene transfer and recombinations may not be relevant. For strain-identification of a bacterial population on a larger scale (non-related samples, separated by time and location), their occurrence is more likely to be expected. When in such studies clonal and stable isolates are nevertheless identified, one could argue that these represent clones of *C. jejuni* that are more stable than others. The reasons for differences in degree of clonality are currently not known.

Conclusions

Population genetics and molecular epidemiology of *C. jejuni* is more complex than it is for other enteropathogens. Although DNA recombinations destroy stability of genotype and DNA transfers destroy clonality, a number of stable clones can still be identified. The occurrence of these phenomena should not be ignored, however their frequency in real-time natural populations can not yet be estimated. Although these biological events complicate the interpretation of data, they should not withhold us to use genotyping techniques for strain identification of *C. jejuni*.

Summary

The application of genetic techniques for strain identification is potentially hampered by frequent occurrence of recombination and transformation events in the *Campylobacter jejuni* population. The consequences of DNA transfer and recombinations to the clonal structure of the population, as determined by MLST, is

discussed. The different mechanisms that can result in instability of genotype, and the consequences to molecular epidemiology is also treated. It can be concluded that recombinations, either occurring with or without DNA transfer, are probably infrequent in small-scale, real-time epidemiological studies. Their accumulative effects become more apparent when bacterial populations are compared in general.

Zusammenfassung

Die Anwendung von gentechnischen Methoden zur Identifizierung von Stämmen wird potentiell behindert durch relativ häufige Rekombinations- und Transformationsereignisse in einer *Campylobacter jejuni*-Population. Die Folgen von DNA-Transfer und Rekombinationen auf die klonalen Strukturen einer Population anhand der MLST-Technik werden diskutiert. Daneben werden auch die verschiedenen Mechanismen, welche zu einer Instabilität des Genotyps führen, behandelt, ebenso die Folgen auf die molekulare Epidemiologie. Daraus kann man schliessen, dass Rekombinationsereignisse – verbunden mit oder ohne DNA-Transfer – wenig vorkommen in kleinen «real-time» Epidemiologiestudien. Akkumulation solcher Effekte wird aber deutlicher sichtbar, wenn bakterielle Populationen im Allgemeinen verglichen werden.

Résumé

L'application des méthodes de la biologie moléculaire pour identifier les souches de *Campylobacter* est potentiellement empêchée par les phénomènes de récombinaisons et de transformation qui se passent dans une population de *Campylobacter jejuni*. Les conséquences du transfert de l'ADN et de la récombinaison sur les structures clonales d'une population, suivie par la technique MLST, sont le sujet de notre discussion. En outre, les différents mécanismes menant à l'instabilité du génome et les conséquences sur l'épidémiologie moléculaire sont traités. Il en résulte que les phénomènes de récombinaison – accompagnés avec ou sans transfert de l'ADN – sont observés rarement dans les études épidémiologique en temps réel. L'accumulation des effets de ce genre est observée quand on compare des populations bactériennes en général.

Key words

Campylobacter, Genome stability, DNA transfer, MLST technique, Molecular epidemiology

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