MOLECULAR METHODS FOR DETECTION, SPECIATION AND SUBTYPING OF CAMPYLOBACTER SPP.

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Introduction

Campylobacter jejuni is one of the most common causes of human enteritis world-wide, and in several European countries it has replaced Salmonellae as the first cause of bacterial enteritis (Notermans, 1994). The strongest epidemiological risk factor for campylobacteriosis identified thusfar is the consumption of poultry products, therefore the problem is of direct concern to the poultry producing industry. There is a growing demand for fast and easy detection of Campylobacter in chickens and poultry products. Recently, molecular methods have been developed that have the potential to replace classical bacteriological methods. Most molecular methods based on the polymerase chain reaction (PRC) are fast, simple, and reliable, however an evaluation of the sensitivity and specificity of the developed detection and speciation methods is needed. The most promising available new methods are summarized here.

It has been recognized for a long time that Campylobacters are extremely diverse in phenotype, and, as became clear more recently, also in genotype. A number of genotyping techniques have been developed for genetic subtyping of Campylobacters. This contribution concentrates on the most common or most promising of these techniques. A selection of the available literature is given here. For a classification of molecular typing methods and for general background information the reader is referred to Vaneechoutte (1996).

Detection and speciation of thermophilic Campylobacter species by molecular techniques

Detection of microorganisms by the polymerase chain reaction has long been predicted as the modern-time alternative for bacteriological culture. However, just as it required over 15 years to identify proper culture methods for Campylobacter (and these are probably still not optimal) it will require time to find the best PCR detection procedure.

In short, PCR is the amplification (multiplication of the amount of DNA) of a specific piece of DNA, making use of specific primers and a DNA polymerase that is extremely thermostable (normally Taq polymerase). The technique is very powerful and can be very sensitive, however, depending on the type of sample, different problems have to be solved. In food products, the low numbers of organisms require pre-enrichment and the presence of potential PCR inhibitors require robust PCR protocols (Thunberg et al., 2000).

Purification procedures to remove PCR inhibitors are effective but add extra work (Wang et al., 1999). In faeces, numbers of Campylobacters may vary and the presence of high numbers of other microorganisms demands a high specificity. In order to detect viable organisms and not killed bacteria, and for higher sensitivity, pre-enrichment may be needed, which greatly reduces the benefit of PCR as a fast one-step method. The issue whether it is desirable to detect viable but not culturable forms is still debated. At the moment there is a tendency to ignore these damaged organisms, since laboratory simulation experiments suggest that colonisation potential is lost before (and not after) culturablilty is lost (Fearnley et al., 1996). However the debate is not yet closed (Cappelier et al., 1999).

Some PCR detection methods developed for Campylobacter spp. detect directly at the species level. Other methods detect C. jejuni and C. coli without differentiation, and in some C. lari is included as well. Most methods are based on amplification of (fragments of) flagellin genes or ribosomal genes. In some described methods the target gene for species-specific amplification has been selected by hybridization experiments and has not been further characterized. A selection of currently available molecular detection methods is summarized in Table 1.

The described methods vary in complexity from a single PCR on direct sample material, to pre-enrichment and/or filtering, amplification, gel electrophoresis followed by Southern blotting (or spot blots) and hybridization. A comparative study including all or even some of these methods is not available to my knowledge. Although some methods are very similar (many use flagellin genes as the target gene but different primers are in use), I am not aware of initiatives for standardization of these molecular detection methods. At present it cannot be concluded which of the described method is superior. In a comparative study speciation by PCR was at least as sensitive as classical biochemical techniques (Steinbrückner et al., 1999).

Speciation within the thermophylic Campylobacters is also possible by molecular methods other than PCR, although sofar none of these have been applied in a large number of laboratories. In one publication Southern blots are hybridized with an unidentified probe that gives size-specific bands for C. jejuni or C. coli (Korolik et al., 1995), but a species-specific PCR is a simpler way to differentiate these. Hybridization of Southern blots with species-specific probes derived from the rRNA genes has also been described (Tenover et al., 1990). The technique known as NASBA ('nucleic acid sequenced based amplification') is based on Taq-independent, room temperature amplification, and this can be used for speciation when it is followed by hybridization (Uyttendaele et al., 1994). Atypical thermophilic Campylobacters can be identified as C . jejuni or C. coli (or neither of these) by slot-blot hybridization (Ng et al., 1987). A more modern approach is by species-specific hybridization of PCR products obtained with degenerate primers (Al Rashid et al., 2000). These described methods still have to prove their value in high-throughput routine laboratories.

Subtyping of Campylobacter spp.

The diversity of biochemical and phenotypic properties within Campylobacter species have been recognized for a long time. In the past, phenotypic differences between isolates were used to develop subtyping schemes. For Salmonella and other enterobacteriacae serotyping had proved valuable, and therefore in the 80's serotyping was developed for Campylobacter (Penner and Hennessy, 1980; Lior et al., 1982). The serotyping scheme based on heat-stable (HS) antigens (Penner and Hennessy, 1980) is still in use in few laboratories and now encompasses

Table 1: A comparison of molecular methods described for detection and speciation of thermophylic Campylobacter spp.

Sample material	Species*	Target gene	Detection limit	Remarks	Reference
PCR dependent methods					
dairy products	jejuni+coli	flaA+B	with raw milk more sensitive than culturing	food samples were treated to liberate bacteria but not enriched	Allmann 1995
stool, water	jejuni+coli	flaA	30-60 CFU/assay in stool, 10-100 CFU/ 100 ml water	for water filtration is required	Oyofa 1992, 1993
chicken litter	jejuni	flaA	with dried litter more sensitive than culturing	enrichment required	Itoh 1995
water	jejuni+coli	flaA+B	10-20 CFU/ml	filtration required	Kirk 1994
water	jejuni	flaA	30 CFU/100 ml	enrichment required	Hernandez 1995
chicken meat washes	jejuni+coli+lari	16S rRNA	25 CFU/g meat	enrichment and hybridization required	Giesendorf 1992
DNA from pure cultures	jejuni+coli+lari+ upsaliensis or jejuni, coli, lari, upsaliensis	23S rRNA	12 CFU/assay	speciation is dependent on choice of primers	Eyers 1993 Fermer 1999
chicken meat	jejuni, coli	rRNA intergenic spacer		hybridization required for speciation	O'Sullivan 2000
cell lysates from pure cultures	jejuni	membrane protein gene mapA	24 CFU/assay gene is also immunogenic	the protein encoded by this	Stucki 1995
DNA from pure cultures	jejuni, coli	hippuricase, aspartokinase		a three-step PCR for detection and speciation	Linton 1997
DNA from pure cultures	jejuni, coli, lari, upsaliensis, arcobacters	glyA	200 CFU/assay	degenerate primers are used for detection, hybridization for speciation	Al Rashid 2000
lysed cells from enriched carcass washes	jejuni	unidentified	not available	filtration, culturing required	Winters 1995
DNA from pure cultures	jejuni	unidentified	1 CFU	hybridization required	Stonnet 1993
DNA from pure cultures	jejuni, coli, lari	unidentified	not available	RAPD PCR followed by hybridization	Giesendorf 1993
PCR independent methods					
pure cultures	jejuni+coli+lari or jejuni, coli, lari	16S rRNA	6 CFU in presence of $4x10^6$ CFU Gram negative bacteria	NASBA followed by hybridization, speciation is dependent on probe	Uyttendaele 1994
pure cultures	jejuni+coli+lari	not specified	not applicable	one-step DNA hybridization	Tenover 1990
DNA from pure cultures	jejuni, coli	not applicable	not applicable	slot blot hybridization to speciate atypical Campylobacters	Ng 1987
DNA from pure cultures	jejuni, coli	unidentified	not applicable	Southern blot hybridization	Korolik 1995

* when multiple species are detected that cannot be differentiated this is indicated by '+'. When a single species is given the method is specific for that species. When the method allows differentiation between species, these are separated by ",".

over 60 serotypes. Other phenotypic subtyping schemes that are still used are summarized in Table 2.
Each of these methods has it's own advantages and disadvantages. The most striking disadvantage of phenotypic subtyping in general is that it depends on expression of a characteristic phenotype, which can be influenced by culture conditions, culture age, etc. Other disadvantages are:

- a relatively high percentage of strains that are untypeable due to lack of phenotypic expression,
- laborious maintenance and quality control of sera and phage collections,
- difficulties in compatibility (see Table 3 for an explanation of the terminology used in this context).

Table 2: A comparison of phenotypic and genotypic methods developed for C. jejuni

Phenotypic methods	Typeability	Discrimination power	Reproducability	Time required	Costs	Specific disadvantages • specific advantages
HS serotyping	80 %	average	aood	<1 day	low	production, maintenance and quality control of sera collection is costly and time consuming • method in use for over 15 years
phage typing	60-80 %	low	good	<1 day	low	loss or change of phagetype is not uncommon
biotyping	data not available	low	low	<1 day	low	outcome can be ambiguous
Genotypic methods	**Typeability**	**Discrimination power**	**Reproducability**	**Time required**	**Costs**	**Specific disadvantages • specific advantages**
fla typing	100 %	reasonable	good	<1 day	low	only one genetic locus is persued which may be genetically instable • can be combined for multiplex PCR
PFGE	100 %	good	good	3-4 days	average	specialized equipment required • most commonly used method at present
ribotyping	data not available	low	good	3-4 days	average	not generally used
RAPD	80 %	average	low	<1 day	low	reproducability between labs problematic
AFLP	100 %	good	good	2-3 days	average	specialized equipment required

Table 3: Explanation of terminology used in this contribution

Term	Explanation	Synonym
discrimination power	the ability to differentiate between genetically unrelated strains	specificity, resolution
reproducability	the ability to identify duplicate samples	reliability
typeability	the percentage of strains tested that give a type	sensitivity
compatebility	the possibility to directly compare the outcome with those from other laboratories	
clone	all offspring of a clone is genetically identical to the ancestor	strain
panmictic	a popuation that is not clonal due to DNA reshuffling via sexual reproduction	
genetic instability	a significant change of genotype in otherwise clonal offspring	

Since most phenotypic characteristics are somehow represented by differences in the genome, genotypic differentiation is a straightforward alternative. The advantages are obvious: genotypes are generally stable and independent on culture conditions or expression of antigens; most genetic methods have higher typeability than phenotypic methods; and computer-aided technology allows excellent compatibility.

A comparison of the currently used genotyping methods is summarized in Table 2. Most methods have good typeability and reproducability. Genotypic methods are diverse and can be devided in methods that depend on a single locus (or several loci) within a genome, and those methods that depend on the complete genome. Some methods rely on the absence or presence of recognition sites for restriction enzymes, other methods are based on PCR amplification. Unfortunately, some - in principal identical - methods are known under different names and different methods share identical or confusing names (see Vaneechootte, 1996).

A brief description of the most common genotypic methods known by their common names and synonyms is given below. The amount of literature is overwhelming and since this has been reviewed recently, references are not included here; instead the reader is referred to Wassenaar and Newell (2000).

Flagellin typing (fla typing)

This method is based on restriction fragment length polymorphism (RFLP) of PCR products derived from the flagellin genes (fla) of C. jejuni. Briefly, fla specific PCR primers are used to obtain a PCR fragment which is digested with restriction enzymes. The obtained banding pattern after agarose gel electrophoresis is determined by the choice of primers and the restriction enzyme used. Fragments are generated in the range of 0.1-1 kilo base pairs (kb). Since C. jejuni contains two flagellin genes

(that are next to each other on the genome), the PCR can detect either one fla gene or two, depending on which primers are used. The primers are designed to bind to strongly conserved sequences (but the sequence inbetween the primers is highly variable) and the primers were found to work for C. coli and C. upsaliensis as well. The different fla typing schemes mainly differ in the choice of primers and enzymes. Typeability can be improved when purified DNA is used instead of cell lysates, but this increases the amount of work. The discriminatory power can be increased by using more than one restriction enzyme. In an attempt to standardize the fla typing schemes, a consensus PCR primer set was proposed (Wassenaar and Newell, 2000). Standardization of enzyme choice and nomenclature is initiated by a European consortium CAMPYNET (Campynet, 1999).

Pulsed Field Gel Electrophoresis (PFGE)

Also known as genomic fingerprinting or macrorestriction profiles. A method based on the presence or absence of recognition sites for restriction enzymes that cut infrequently in the genome. PFGE is dependent on complete chromosomal DNA, which is isolated in a protective gelling agent to avoid shearing. After digestion the DNA is analyzed on agarose gels using specialized equipment that generates a pulsing electrical field. In this way large (20-200 kb long) fragments can be separated. The obtained banding pattern depends on the choice of restriction enzyme and the electrophoretic conditions, both of which are in need of standardization (Campynet, 1999).

PFGE is one of the most commonly used methods and is often presented as a 'gold standard' for genotyping, although there is no clear advantage of this method over others, and the method is rather laborious. Some strains are not typeable because of DNAse production, which can be overcome by adaptation of the method. Discriminatory power can be increased by the use of more than one restriction enzyme. A small percentage of strains have DNA that is undigestable by commonly used enzymes, presumably by restriction/modification systems. Such strains are sometimes typeable using alternative enzymes.

Ribotyping

This method is based on the presence or absence of restriction sites in or around the three ribosomal loci, which are visualized by Southern blot hybridization. Briefly chromosomal DNA is isolated, digested, and separated on agarose gels. From these gels a Southern blot is obtained (a 'blueprint' of the separated DNA bands on a nitrocellulose filter) which is hybridized with a labeled DNA fragment specific for the ribosomal RNA (rRNA) genes. The rRNA specific labeled fragment (the 'probe') is usually produced by PCR. The obtained banding pattern depends on the choice of restriction enzymes and the choice of the labeled fragment, which can be obtained from the genes encoding 16S rRNA, 23S rRNA, or both. The method is rather laborious and the discriminatory power is relatively low. The reason for this is not completely understood.

In comparison to other species (e.g. Salmonella), where ribotyping proved to have excellent discriminatory power, C. jejuni contains less ribosomal gene loci (3 as compared to 5 for Salmonella). The fragments detected by ribotyping are 0.5 - 5 kb and the resolution of the gels is poorer than gels used for fla typing. Ribotyping has not been used as much as fla typing or PFGE. An automatic device (commercially available under the name 'riboprinter') allows high throughput with little handling, at high costs for equipment and materials.

Random Amplyfied polymorphic DNA (RAPD)

This method also called Arbitrarily Primed PCR fingerprinting (AP-PCR), is based on PCR but does not amplify specific loci. Instead, arbitrary developed primers are used to amplify randomly distributed fragments. The amplification conditions are chosen at low stringency so that fragments can be amplified even when the primers do not perfectly fit. The obtained PCR products are separated by agarose gel electrophoresis and the obtained patterns (consisting of bands with varying intensity) depend on the presence, orientation, and location of primer sites.

The major problem with RAPD is the lack of reproducability. The low stringency required for the PCR makes the method very sensitive to experimental conditions (purity and concentration of the DNA, inhibitors, PCR apparatus, etc.). The original method used one primer but variants have been described for Campylobacter using two primers, one of which may be specific for enterobacterial repetitive sequences (REP primers). A classical REP-PCR amplifies fragments between repetitive sequences to which REP primers bind with high specificity. Since such repetitive sequences are absent in Campylobacter, a classical REP-PCR cannot be used and the primers are used at low stringency instead, which resembles RAPD. The lack of reproducibility greatly limits compatability of the method, and therefore RAPD is mainly in use in individual laboratories, where good results are reported. The method is fast and simple.

Amplified fragment length polymorphism (AFLP)

This genotyping method should not be confused with methods that determine the size of bands of PCR products (PCR RFLP), which is known under the same name. In AFLP a combination of PCR amplification and restriction enzyme recognition is used in a relatively complex way. Chromosomal DNA is isolated and digested with two restriction enzymes that cut relatively frequently. After ligation of linkers, a subset of these fragments are amplified by PCR in an ingenious way in which the restriction sites serve as the primer-specific sequences, with the addition of one or more specific nucleotides.

The difference with PFGE is that the obtained fragments are much smaller (50-500 bp) and that they are analyzed on acrylamide gels at very high resolution. The difference with RAPD is that the PCR reaction is carried out under stringent conditions which results in high reproducability. The method is relatively new but compatibility proved to be high. Automated gel reading and data processing by computer has greatly aided to objective interpretation of the results, and the high number of generated bands gives a certain leeway in band variation due to artefacts that are averaged out. AFLP has excellent typeability and discrimination power, and may well become the 'gold standard' of the future. However specialized equipment for acrylamide electrophorese and automated gel-reading is needed and the method is not fast, although the throughput is reasonable.

Other methods

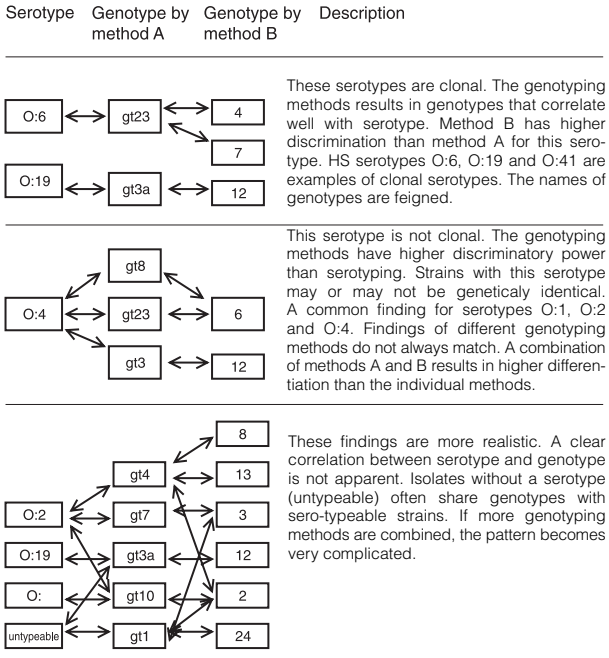
Several genotyping methods have recently been developed, or applied, to Campylobacter. Most of these are variations of PCR RFLP, which means they are similar to fla typing but use other genes as targets. A promising development is to combine such target genes in a multiplex PCR (Ragimbeau et al., 1998; Denis et al., 1999), which highly increases the discriminatory power as compared to the individual PCR RFLP methods. A different approach is multi-locus sequencing (MLST). The latter method is the best to determine the genetic relationship of different lineages and clones, but is not optimal for epidemiological studies.

Genotyping and phenotyping methods compared

The discrimination power of genotyping methods is usually better than that of phenotypic methods, which is reflected in the higher number of different subtypes that can be obtained. An advantage of genotypic data over phenotypic data is that they can be used for phylogenetic analysis, so that the relative genetic relationship between different subtypes can be examined. This enables us to recognize not only THAT two isolates are different, but also HOW different they are. That is not possible with phenotyping.

All of the methods listed here have been applied to compare isolates of different sources (human, animal and sometimes environmental), and all methods tested were able to differentiate outbreak strains from non-related isolates. Several studies compared different genotyping methods in terms of discriminatory power and typeability, and many studies compared these relatively to HS serotyping as well. The most striking finding was that serotypic data and genotypic data, obtained with different methods, do not always match. This is illustrated in Figure 1.

Figure 1: Schematic representation of a comparison of results obtained with serotyping and two independent genotyping methods.



The reason for the lack of correlation between serotyping and genotyping is that some serotypes are clonal (all isolates belonging to that serotype are genetically identical or near to identical); whereas others are not clonal, but panmictic. Non-clonal populations can be formed by sexual reproduction, where DNA fragments of different strains recombine. In this way the genetic loci for the HS antigens can be present in strains of otherwise different genetic makeup so that the serotype is no longer linked to genotype (as determined by methods independent of HS genetic loci). For those serotypes that are clonal, a given serotype will correlate with a given genotype if the two methods have comparable discriminatory power. A genotyping method with higher discriminatory power will discriminate isolates of identical serotype.

When two genotyping methods are compared, the method with the highest discrimination will divide an apparent homogeneous genotype group as determined with a method of low discrimination. For a panmictic population there will be no correlation between serotype and genotype, or between genotypes determined by different methods (figure 1). Of all genotyping methods, AFLP seems to result in the best correlation between serotype and genotype (B. Duim, J.A. Wagenaar and T.M. Wassenaar, unpublished data) and is most suitable for phylogenetic analysis. Probably this is so because AFLP combines a high discriminatory power with a high number of bands from all over the genome, which allows reliable phylogenetic analysis.

Genetic instability

It is obvious that genotypes of bacterial isolates have to remain stable over time to be of use. Fortunately this is nearly always the case. Genotypes do not change when isolates are stored, cultured, or passed in vivo. In exceptional cases, however, genetic instability (a change of genotype in otherwise clonal offspring) of Campylobacters has been reported, and could be detected by various genotypic methods (Wassenaar et al., 2000). For instance, under laboratory conditions it is possible that complete fla types are exchanged between strains, so that a correlation between fla type and the rest of the genome is lost, or that recombinations within the fla locus result in a change of fla genotype in what is otherwise clonal offspring (Wassenaar et al., 1995). It remains to be investigated if and how frequent such events take place under natural conditions. It has also been observed that PFGE genotypes can change within a clonal lineage due to recombinations, insertions, deletions, and point mutations (for references see Wassenaar et al., 2000). Again, the frequency of such events is not known and may differ from strain to strain. Depending on the frequency, such events may not be of importance for short-term epidemiology, such as horizontal spread on a chicken farm, or identifying potential contamination sources. However, for long-term studies it is advised to combine two independent techniques, either genotypic or phenotypic (or a combination of both), with sufficient discriminatory power to correct possible effects of genetic instability. The use of two methods also compensates a possible lack of discrimination of single methods. In Wassenaar et al. (2000) examples are described how one can recognize and differentiate results due to genetic instability from observations that result from the presence of unrelated, different genotypes.

Concluding remarks

The methods described in the literature for molecular detection and speciation of Campylobacter spp. are

promising but further evaluation and comparison is needed to select the best method in a practical setting. Since molecular detection is fast and reliable it is expected that their application in routine laboratories will rapidly increase. Molecular typing has already served it's value in epidemiological studies with large numbers of samples (Lawson, 1999).

Standardization of the genotyping techniques is needed to allow compatibility between laboratories. Most subtyping genetic methods require culturing and/or DNA isolation, and thus cannot be directly combined with molecular detection. The flagellin gene could in theory be target for both detection and subtyping. Genetic subtyping can reveal which subpopulations of bacteria are mainly found in chicken and poultry products, and whether these differ from the subpopulations seen in humans and in the environment. Subtyping can further be applied to identify contamination sources and thus help in implementing effective intervention strategies.

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