(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 February 2015 (12.02.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/020671 A1

(51) International Patent Classification:
C12Q 1/68 (2006.01) *C07H 21/04* (2006.01)
C12P 19/34 (2006.01)

(21) International Application Number:
PCT/US2013/054309

(22) International Filing Date:
9 August 2013 (09.08.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
13/963,146 9 August 2013 (09.08.2013) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: MULTIPLEX AMPLIFICATION REACTION METHOD FOR DETERMINATION OF CAMPYLOBACTER JEJUNI PENNER/CAPSULE TYPE

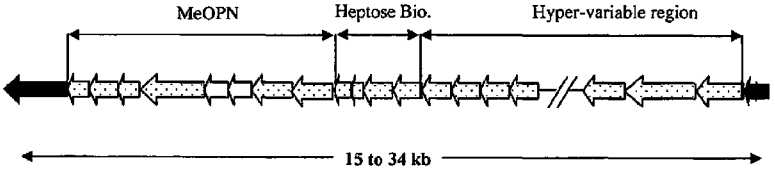

FIG 1

(57) Abstract: The inventive method and associated reagents relate to a molecular approach to determining Campylobacter jejuni capsule/Penner types. The invention also relates to a method of identifying Campylobacter jejuni types using primers in a multiplex PCR assay.

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MULTIPLEX AMPLIFICATION REACTION METHOD FOR DETERMINATION OF CAMPYLOBACTER JEJUNI PENNER/CAPSULE TYPE

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. nonprovisional application no. 13/963,146, filed 9 August 2013, which is a Continuation-in-Part of U.S. nonprovisional application no. 13/031,718, filed 22 February 2011, which claims the benefit of U.S. Provisional Application No. 61/307,632, filed 24 February 2010, which are incorporated by reference, herein.

BACKGROUND OF INVENTION

1. Field of Invention

[0002] The inventive subject matter relates to a molecular method for determining *Campylobacter jejuni* capsule/Penner types.

2. Background

[0003] *Campylobacter* is a major cause of human bacterial diarrheal disease worldwide, with *C. jejuni,* and to a lesser extent *C. coli,* the most important pathogenic *Campylobacter* species. Campylobacteriosis symptoms range from asymptomatic infection to bloody diarrhea associated with abdominal pain and fever. The major source of human infection is through consumption of uncooked poultry, which is commonly colonized by *C. jejuni.* Post infectious sequelae associated with *C. jejuni* include reactive arthtitis, Guillain-Barré syndrome and irritable bowel syndrome.

[0004] The molecular pathogenesis of C. *jejuni* is not well understood, but a polysaccharide capsule (CPS) is one of the few recognized virulence determinants of this pathogen. The capsular polysaccharide undergoes a reversible phase variation in expression (Bacon, *et al.*,Mol. Microbiol. 40:769-777 (2001)). The capsule contributes to serum resistance of C. *jejuni,* the ability of C. *jejuni* to invade intestinal epithelial cells *in vitro*, and, in a ferret model, is required for virulence (Bacon, *et al.*,Mol. Microbiol. 40:769-777 (2001)). More recently, polysaccharide capsule conjugated to a protein carrier has been shown to protect non-human primates against diarrheal disease (Monteiro, *et al.*, Infect Imm.77(3): 1128-36 (2009)). Differentiation of *Campylobacter jejuni* strains is typically conducted through the use of Penner serotyping.

[0005] The Penner or "heat stable" serotyping scheme is a passive slide hemaglutination assay for both *C. jejuni* and *C. coli* that includes 47 *C. jejuni* serotypes. Rabbit polyclonal antibodies are generated against whole cells of each of the 47 type strains. Antigens are extracted from *C. jejuni* strains to be tested by heating bacterial suspensions in saline at 100°C. These "heat-stable" antigens are used to sensitize sheep erythrocytes, which are used in a passive slide hemagglutination assay with the specific polyclonal antisera. Genetic studies indicate that CPS is the major serodeterminant of the Penner scheme. Thus, mutation of genes required for CPS biogenesis rendered many strains un-typable in the Penner scheme.

[0006] However, other surface heat stable surface structures such as lipooligosaccharides (LOS) may also contribute to serospecificity of some Penner types. The capsular polysaccharides of *C. jejuni* are known to be structurally diverse (Karlyshev *et al.*, *Molecular Microbiology* 55:90-103)(2005)). This structural diversity is consistent

with the variability observed in the genes encoding the capsule in *C. jejuni*. The capsule locus of *C. jejuni* includes both highly conserved genes involved in capsule synthesis and highly variable loci that encode genes involved in synthesis of specific sugars and specific glycosyl transferases required to link the sugars together. The variable CPS locus located between two conserved genes, *kpsC* and *kpsF*, and the variable genes can range from 15 to 34 kb (FIG 1). Variable genes also encode synthesis and transfer of modifications to the sugars, such as methyl phosphormidate (MeOPN) (Karlyshev *et al.*, *Molecular Microbiology* 55:90-103).

[0007] Penner serotyping is technically difficult to perform and expensive to produce the type antisera. As a result, only a handful of reference laboratories routinely perform Penner typing. Moreover, many serotypes fall into Penner "complexes". The significance of these complexes is not totally understood in most cases, but they appear to include capsules with related structures (Aspinall, *et al.* Carbohydr Res. 231:13-30 (1992)).

[0008] Others have tried to replace the laborious Penner serotyping using a molecular typing approach involving restriction fragment length polymorphism (RFLP) analysis of PCR amplified lipooligosaccharide (LOS) loci (Shi *et al.* J Clin Microbiol. 40(5):1791-7 (2002); Nakari *et al.*, J Clin Microbiol. 43(3):1166-70) (2005)). However, these RFLP methods have not been widely used and have not replaced Penner serotyping as the typing method of choice. This may be due in part to the RFLP method requiring amplification of a 9.6kb fragment. Using PCR to generate such large amplicons is difficult and can place special requirements on the PCR conditions and reagents used, as demonstrated by Nakari *et al.*, who were unable to generate amplified fragments using

the amplification conditions described by Shi, *et al.* These RFLP methods are also limited because they are based on the amplification of the LOS locus. At the time of the Shi et al. study, it was known that both the LOS and CPS structure were part of the Heat Stable antigen (HS) recognized through the Penner serotyping method. However, in 2005, CPS was demonstrated to be the major serodeterminant of the Penner method (Karylshev, *et al.*, Mol. Micro. 55: 90-103 (2005)). This helps explain why Shi et al. and Nakari et al. found only partial correlation between the Penner serotypes and RFLP groups. Penner serotyping distinguishes strains that cannot be distinguished by this RFLP method. For example, the most common RFLP type, Hh1Dd1, contained strains belonging to several HS serotypes, including HS 6,7, HS 12, HS 27, HS 55, HS 21, HS 10, HS 57, HS 6, HS 15, HS 23,36,53, and HS 27 + HS 31 (Nakari *et al.*, J Clin Microbiol. 43(3):1166-70 (2005)). And some serotypes, such as HS 2, HS 3, HS 4 complex, HS 8, HS 10, HS 11, HS 12, HS 15, HS 19, HS 31, HS 32, HS 41, HS 57, and HS 23,36,53 include more than one RFLP (Nakari *et al.*, J Clin Microbiol. 43(3):1166-70 (2005)).

SUMMARY OF THE INVENTION

[0009] The current invention relates to reagents and method to identify *Campylobacter jejuni* Capsule/Penner types via molecular, rather than serological, methods.

[0010] Therefore, an object of the invention is a panel of multiplex DNA primers for identification of *C. jejuni* Capsule/Penner types by polymerase chain reaction (PCR).

[0011] Several important advantages of amplification reactions over serological determination are evident. First, it is technically difficult to perform and expensive to

produce type antisera. As a result, few reference laboratories are capable of routine Penner typing. Additionally, many serotypes fall into Penner "complexes."

[0012] Amplification methods, unlike typing sera methods, are relatively available to research and reference laboratories. Furthermore, no expression of capsule is needed. Therefore, there are no affects due to phase variation in capsule expression, as is possible with serotyping. Multiplexing reduces the number of reactions to be performed per samples. Additionally, amplification reactions do not suffer from CPS being shut down or modified thru slipstrand mutations. The instant invention can identify 23 serotypes.

[0013] The multiplex amplification technique amplifies a fragment less than 1 kb that can be routinely performed in any molecular biology lab worldwide.

BRIEF DESCRIPTION OF DRAWINGS

FIG 1. Schematic of the general organization of the capsule loci of C. jejuni. The region between kpsC and kpsF (black arrows) encodes the genes for synthesis of distinct capsule structures. If present, genes for heptose and MeOPN synthesis are highly conserved. The region to the right is the hyper-variable region containing sugar transferases and sugar biosynthetic genes.

FIG 2. Predicted product size for amplicons. Amplified DNA is separated and sized through an agarose gel (2%), run in 0.5 x TBE buffer. The underlined products are those defined by the primers in this current application. The other products are identified by the PCR primers in U.S. Patent application no. 13/031,718, filed 22 February 2011, to which this application claims priority.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0014] The following terms are defined:

[0015] "Amplification reaction" refers to a method of detecting target nucleic acid by *in vitro* amplification of DNA or RNA.

[0016] "Polymerase chain reaction (PCR)" refers to the amplification of a specific DNA sequence, termed target or template sequence, that is present in a mixture, by adding two or more short oligonucleotides, also called primers, that are specific for the terminal or outer limits of the template sequence. The template-primers mixture is subjected to repeated cycles of heating to separate (melt) the double-stranded DNA and cooling in the presence of nucleotides and DNA polymerase such that the template sequence is copied at each cycle.

[0017] "Primer" refers to DNA oligonucleotides complementary to a region of DNA and serves as the initiation of amplification reaction from the 5' to 3' direction.

[0018] "Primer pair refers to the forward and reverse primers in an amplification reaction leading to amplification of a double-stranded DNA region of the target. PCR primer "mix" is defined as the forward and reverse primer pairs for specific targets, whereby the products within the "mix" differ by at least 20 bp.

[0019] "Target" refers to a nucleic acid region bound by a primer pair that is amplified through an amplification reaction. The PCR "product" or "amplicon" is the amplified nucleic acid resulting from PCR of a set of primer pairs.

[0020] The term "multiplex amplification reaction" herein refers the detection of more than one template in a mixture by the addition of more than one set of

oligonucleotide primers. In a preferred embodiment, primer pairs are grouped into "mixes" to ensure ready detection of PCR products.

[0021] The term "capsule" herein refers to the structure lying outside the cell wall of bacteria, such as *Campylobacter jejuni*.

[0022] Utilizing genomic and capsule loci sequences, a molecular method for determining Penner and capsule type was developed. This method is simpler than Penner serotyping. The inventive method is more easily standardized than Penner serotyping, since molecular reagents (i.e., primers) can be produced and standardized resulting in lower cost. Additionally, the method does not require that the capsule be expressed. Therefore, it is not affected by phase variation in capsule expression, unlike the typing system.

[0023] In one embodiment, the current invention provides a method to specifically distinguish specific *C. jejuni* strains and recognize Capsule/Penner serotypes thru PCR amplification of type specific sequences. The inventive method and reagents permit identification of *Campylobacter jejuni* Penner types without the potential for capsule shutdown or modification due to slip-strand mutations.

Example 1: PCR primers correlating to Penner serotype

[0024] The capsule locus of C. *jejuni* includes both highly conserved genes involved in capsule synthesis and highly variable loci that encode genes involved in synthesis of specific sugars and specific glycosyl transferases required to link the sugars together. The variable CPS locus, located between two conserved genes, *kpsC* and *kpsF*, and the variable genes range from 15 to 34 kb (FIG 1). Variable genes also encode synthesis and

transfer of modifications to the sugars, such as methyl phosphoramidate (Karlyshev, A. *et al.*, Mol. Microbiol. 55:90-103 (2005)). In a preferred embodiment, based on the DNA sequences, unique DNA sequences from the capsule loci (FIG. 1) of *C. jejuni*, for each Penner type, were identified. The selected genes were further compared to the whole genome sequences of *C. jejuni* in order to eliminate potential similarities with genes outside the CPS region.

[0025] Selection of genes unique to a particular serotype was performed using a local BLAST program. Each single gene of the variable capsule region (between *kpsC* and *kpsF*) was compared with a database containing the nucleotides sequences of all the available capsule loci of *C. jejuni*. The selected genes were further compared to the whole genome sequences of *C. jejuni* sequenced genomes to eliminate potential similarities with genes outside CPS region.

[0026] Multiplex primers were designed using PCR primers capable of correcting errors and closing gaps. Development of unique Campylobacter *jejuni* PCR primer sequences were undertaken by sequencing DNA of capsule loci derived from the strains: HS19, HS33, HS63, HS57, HS12, HS27, HS21, HS31, HS62, HS45, HS29, HS22, HS9, HS37, HS18, HS58, HS52, HS60, HS55, HS32, HS11, HS40, HS38, HS7, HS31, HS35, HS16, HS43, HS50, HS64 and HS65.

[0027] CPS sequencing strategies was undertaken within the conserved heptose genes hddA and dmhA region. If the strains to be sequenced produced a positive amplification with primers for hddA and dmhA, these genes were used as anchors for long-range PCR. This two-step PCR increased the probability of amplification by lowering the size of the PCR product. PCR amplifications were performed using a MASTERAMP™ Extra-Long

PCR kit from Epicentre (Madison, WI) or LONGAMP™ Taq DNA polymerases (New England Biolabs, Ipswich, MA). CPS locus sequences were obtained by cloning the *kpsC-hddA* and *KpsF-dmhA* PCR fragments into a pCR4-TOPO™ vector (Invitrogen, Carlsbad, CA) in order to create a representative genomic library. Following purification, clones were sequenced. Assembly was performed using SEQUENCHER® 4.8 (Gene Codes Corporation, Ann Arbor, MI).

[0028] A database of CPS loci was created to identify unique regions of each serotype. PCR primers, using online software, were designed with the following parameters: length between 18 and 30 residues, 20 to 50% GC content, and melting temperature ranging from 57 to 63° C. The primer sequences were verified for absence of dimerization or hairpin formation using AUTODIMER™ (Vallone and Butler, Biotechniques 37(2): 226-231 (2004)). The PCR primer sets (i.e., forward and reverse primers) were grouped into multiple mixes so that each group or mix produced amplicons that differ by at least 20 bp from the other amplicons in the same group or mix.

[0029] The forward and reverse primers are shown in Table 1, along with the associated sequence identity number (SEQ ID No.). The primers were designed within genes within the CPS loci and are summarized in Table 1. Table 1 identifies the sequence identification numbers (SEQ ID No.) of the forward and reverse primers along with the product size.

Table 1

Mix Alpha	PCR Product size (bp)	Penner type identified	Designed in Gene (function)	Forward primer (SEQ ID No.)	Reverse primer (SEQ ID No.)	PCR Product SEQ ID No.
Mu_HS19	450	HS19	HS19.07 (MeOPN transferase)	1	2	47
Mu_HS63A	522	HS63	HS63.23 (glycosyl transferase)	3	4	48
Mu_HS33A	819	HS33 and HS35	HS33.07 (MeOPN transferase)	5	6	49
Mix Beta						
Mu_HS57	100	HS57	HS57.02 (Unknown)	7	8	50
Mu_HS12D	201	HS12	HS12.15 (glycosyl transferase)	9	10	51
Mu_HS27A	280	HS27	HS27.12 (sugar transferase)	11	12	52
Mu_HS21A	801	HS21	HS21.05 (NAD-dep. epimerase/dehydratase)	13	14	53
Mu_HS31	857	HS31	HS31 17-18 (RmlD (RmlD substrate binding domain protein)	15	16	54
Mix Gamma						
Mu_HS62	82	HS62	HS62.09 (Unknown)	17	18	55
Mu_HS45A	128	HS45	HS45.10 (dmhA)	19	20	56
Mu_HS29A	185	HS29	HS29.07 (MeOPN transferase)	21	22	57
Mu_HS22G	216	HS22	HS22.08 (sugar transferase)	23	24	58
Mu_HS9A	278	HS9	HS9.08 (sugar transferase)	25	26	59
Mu_HS37	541	HS37	HS37.28	27	28	60
Mu_HS18A	653	HS18	HS18.07	29	30	61
Mix Delta						
Mu_HS58C	85	HS58	HS58.13 (sugar transferase)	31	32	62
Mu_HS52C	170	HS52	HS52.07 (MeOPN transferase)	33	34	63
Mu_HS60A	241	HS60	HS60.14 (Unknown)	35	36	64
Mu_HS55B	341	HS55	HS55.06 (Unknown)	37	38	65
Mu_HS32A	420	HS32	HS32.18 (GDP-fucose protein O-fucosyltransferase)	39	40	66
Mu_HS11D	540	HS11	HS11.11 (Unknown)	41	42	67
Mu_HS40C	636	HS40	HS44.13 (transketolase)	43	44	68
Mu_HS38B	741	HS38	HS38.05 (CMP-KDO synthetase)	45	46	69

[0030] Comparison of CPS loci resulted in confirmation that the relation between strains belonging to the same complex had similar CPS loci. For example, the strain

HS33 CPS loci is highly similar to HS35. No difference of CPS sequence was identified between these serotypes. As such, PCR primers that identify HS33 also identify HS35 and HS33/35 strains (i.e., defined as the HS33 complex). Similarly, HS5 is highly similar to HS31, with no difference of CPS sequence identified between theses serotypes. Consequently, HS5 and HS31 are also associated using Penner serotyping. Therefore, PCR primers that identify HS31 also identify HS5 and HS5/31 strains (i.e., defined as the HS5 complex). Also, HS6 is similar to HS7. HS6 and HS7 are also associated using Penner serotyping. No difference in CPS nucleotide sequence was found between these serotypes. As such, Mu_HS6 primers identify HS6, HS7 and HS6/7 strains (defined as HS6 complex).

Example 2: Multiplex PCR assay

[0031] In a preferred embodiment, PCR primers were designed in regions that were found unique to each particular *C. jejuni* serotype. In a preferred embodiment, the PCR primers were designed to permit multiplex PCR. Multiplex PCR significantly reduces the number of reactions needed for strain identification. Design of the multiplex primers was conducted utilizing the online software MUPLEX^{Tm} (Boston University, Boston, MA) (described in Rachlin, et al., Nucleic Acid Research 33 (Web Server Issue): W544-W547) (2005).

[0032] In one embodiment, primer sets are grouped into multiple "mixes" based on the sizes of the products amplified. The amplified products (i.e., amplicons) for each primer pair is shown in Table 1, along with the associated sequence identification number (SEQ ID NO.). In a preferred embodiment, amplification and identification of *C. jejuni*

strains is conducted utilizing four (4) "mixes" or groupings: (alpha) α; (beta) β; (gamma) γ; and (delta) Δ mixes, although other potential groupings or mixes are contemplated. The "mixes" or groupings of primer pairs, along with the associated *C. jejuni* strain(s), in the preferred embodiment, is illustrated in Table. 1. In Table 1, the alpha mix contains primers that distinguish HS19, HS63, and HS33/HS35. The beta mix contains PCR primers that distinguish HS57, HS12D, HS27A HS21A and HS31. The gamma mix contains primers that can distinguish strains HS62, HS45, HS29, HS22, HS9, HS37 and HS18. The delta mix contains PCR primers that can distinguish *C. jejuni* strains HS58, HS52, HS60, HS55, HS32, HS11, HS40 and HS38.

[0033] Primers were evaluated for their ability to enable efficient amplification of *C. jejuni* target DNA, resulting in a predicted product and for not interfering with other primers included in the reaction. The primer sets for a given "mix" were designed to produce amplicons that differ by at least 20 bp from the other amplicons in the same mix. Primer sets were judged satisfactory if they produced the expected size PCR product on their Penner serotype DNA template or related complexes and were negative for other tested serotypes. A positive control is also included to control assay operation and to evaluate whether the samples are derived from *C. jejuni*. The positive control is included in the "mix" that, like the other primer pairs, results in a difference of at least 20 bp from the other primers. In a preferred embodiment, the control are IpxA primer sets, although other controls are contemplated.

[0034] Although other potential PCR parameters are contemplated, in a preferred embodiment, the PCR amplification of *C. jejuni* samples comprises the following steps:

a. Obtain a sample suspected of containing *Campylobacter jejuni* DNA;

b. Subject sample containing said DNA to one or more of the primer pairs listed in Table 1, or a primer pair capable of amplifying the same product shown in Table 1. In a preferred embodiment, the primers are 18-30 nucleotides, have a G/C content of 20-50%, and a melting temperature between about 57°C and 63°C;

c. Amplify target DNA under the following parameters: 94°C for 30", 56°C for 30", 72°C for 45" for a total of 29 cycles;

d. Subsequent to PCR amplification compare PCR product size.

[0035] Amplifying DNA from an unknown *C. jejuni* sample, using the primers in Table 1, and comparing the size of the ensuing amplification products permits identification of *C. jejuni* Penner serotypes. In a preferred embodiment, the amplified DNA is separated and sized. In one embodiment, sizing is through an agarose gel (2%), run in 0.5 x TBE buffer. The sizes of the PCR products and corresponding serotype are determined by comparison with 100 bp molecular size standards. In a preferred embmodiment, a positive control is included. As an example, primers to the gene IpxA is used as a control. In a preferred embodiment, the IpxA control is included in the gamma (γ) mixture to ensure the easiest visualization of the predicted 331 bp product. If the sample was derived from *C. jejuni*, a 331 bp product should be observed. If no 331 product is obtained, then errors were made in the application of the assay method or the sample is not derived from *C. jejuni*. Although agarose gel electrophoresis is a preferred method, other methods to analyze PCR product size are contemplated.

[0036] FIG. 2 illustrates the product migration by agarose gel electrophoresis (2% agarose) and the associated strains. The capsule loci sequences obtained were then compared to Penner serotyping results. The predicted PCR product size, for a given "mix" and associated Penner serotype is illustrated in the results shown in FIG. 2. In the example illustrated in FIG. 2, the primers are grouped into an α, β, γ, and Δ "mix", based on achieving at least 20 bp difference between the PCR products, in order to easily distinguish products.

[0037] In other embodiments, methods are carried out, at least in part, using a solid support. A variety of different supports can be used. In some embodiments, the solid support is a single solid support, such as a chip or wafer, or the interior or exterior surface of a tube, cone, plastic plate or other article. In some embodiments, the solid support is a particulate support, also referred to as a microsphere, bead or particle. Typically, the particles form groups in which particles within each group have a particular characteristic. Examples of suitable characteristics include, but are not limited to, color, fluorescence frequency, density, size, or shape. The selection of characteristics will depend on multiple criteria including the ability to distinguish or separate target-bound particles from particles of other groups. Particles can be separated by a number of methods. In a preferred embodiment, the particles can be separated using techniques, such as, for example, flow cytometry.

[0038] The particles can be fabricated from virtually any insoluble or solid material. For example, the particles can be fabricated from silica gel, glass, nylon, resins, SEPHADEX™, SEPHAROSE™, cellulose, magnetic material, a metal (e.g., steel, gold, silver, aluminum, copper, or an alloy) or metal-coated material, a plastic material (e.g.,

polyethylene, polypropylene, polyamide, polyester, polyvinylidenefluoride (PVDF)) and the like, and combinations thereof. Examples of suitable micro-beads are described, for example, in U.S. Patent Nos. 5,736,330, 6,046,807 and 6,057,107, all of which are incorporated herein by reference in their entirety.

[0039] Thus, in one embodiment, the multiplex method described herein is performed using microspheres conjugated to unique capture oligonucleotides, permitting the analysis of many different nucleic acids in a single reaction. Each unique capture oligonucleotide is complementary to a unique tag sequence within one of the amplicons to be detected. In this embodiment, the microsphere mix consists of a number of microspheres equal to the number of serotypes that can be detected in the assay. Each of the microspheres contains a different fluorescent dye mix and is coupled to a unique capture oligonucleotide sequence complementary to a unique tag sequence within the amplicon of each serotype of interest. The hybridization of the capture oligonucleotide and the tag sequence of an amplicon results in the coupling of the amplicon to the solid support. The unique capture oligonucleotide and its complementary tag sequence are, thus, associated with a single, specific Penner serotype. The capture oligonucleotides are designed so there is no cross-hybridization between the capture oligonucleotides and the amplicons from more than one serotype under the hybridization conditions used.

[0040] In this method, the multiplex primer sets are used to amplify regions of interest in a *C. jejuni* DNA sample in the presence of a biotinylated dNTP mixture. Instead of running the amplified PCR fragments on an agarose gel to estimate their size, the amplified PCR fragments are incubated with microspheres conjugated to capture

oligonucleotides specific for the serotypes of interest and streptavidin conjugated to a dye, such as phycoerythrin, and analyzed using an appropriate detection system.

[0041] Having described the invention, one of skill in the art will appreciate in the appended claims that many modifications and variations of the present invention are possible in light of the above teachings. It is therefore, to be understood that, within the scope of the appended claims, the invention may be practices otherwise than as specifically described.

What is claimed is:

1. A method of identifying *Campylobacter jejuni* strains in a sample suspected of containing *Campylobacter jejuni* DNA by polymerase chain reaction, wherein the amplification products of said polymerase chain reaction are derived from genes within the *Campylobacter jejuni* polysaccharide capsule (CPS) loci, comprising: (a) subjecting DNA from said sample to a PCR amplification reaction using one or more PCR primer pairs targeting one or more regions of the *C. jejuni* O-methyl phosphoramidate synthesis region, heptose synthesis and hyper-variable region of the *Campylobacter jejuni* polysaccharide capsule loci; (b) analyzing amplification products resulting from said amplification reaction.

2. The method of claim 1, wherein said polysaccharide capsule loci is derived from *Campylobacter jejuni* strains selected from HS19, HS63, HS33, HS35, HS57, HS12, HS27, HS21, HS31, HS62, HS45, HS29, HS22, HS9, HS37, HS18, HS58, HS52, HS60, HS55, HS32, HS11, HS40, and HS38.

3. The method of claim 1, wherein said amplification products are analyzed by size determination.

4. The method of claim 1, wherein said PCR primer pairs contain sequences selected from the group consisting of: SEQ ID No. 1 and SEQ ID No. 2; SEQ ID No. 3 and SEQ ID No. 4; SEQ ID No. 5 and SEQ ID No. 6; SEQ ID No. 7 and SEQ ID No. 8; SEQ ID No. 9 and SEQ ID No. 10; SEQ ID No. 11 and SEQ ID No. 12; SEQ ID No. 13 and SEQ ID No. 14; SEQ ID No. 15 and SEQ ID No. 16; SEQ ID No. 17 and SEQ ID No. 18; SEQ ID No. 19 and SEQ ID No. 20; SEQ ID No. 21 and SEQ ID No. 22; SEQ ID No. 23 and SEQ ID No. 24; SEQ ID No. 25 and SEQ ID No. 26; SEQ ID No. 27 and SEQ ID

No. 28; SEQ ID No.29 and SEQ ID No. 30; SEQ ID No. 31 and SEQ ID No. 32; SEQ ID No. 33 and SEQ ID No. 34; SEQ ID No. 35 and SEQ ID No. 36; SEQ ID No. 37 and SEQ ID No. 38; SEQ ID No. 39 and SEQ ID No. 40; SEQ ID No. 41 and SEQ ID No. 42; SEQ ID No. 43 and SEQ ID No. 44; and SEQ ID No. 45 and SEQ ID No. 46.

5. The method of claim 1, wherein said PCR reaction is multiplex amplification reaction.

6. The method of claim 1, wherein said primers are grouped in an alpha mix and a beta mix with the alpha and beta mixes that are separately added to an unknown DNA sample in order to discriminate product sizes.

7. The method of claim 1, wherein said sample is a clinical sample.

8. The method of claim 1, wherein said sample is collected from a matrix selected from the group consisting of a bacterial culture, a blood, a tissue, and fecal material.

9. The method of claim 1, wherein the primers have about 18-30 nucleotides, a G/C content of 20-50%, and a melting temperature between about 57°C and 63°C.

10. The method of claim 1, wherein said amplification reaction yields one or more amplification products selected from the group consisting of SEQ ID No. 47; SEQ ID No. 48; SEQ ID No. 49; SEQ ID No. 50; SEQ ID No. 51; SEQ ID No. 52; SEQ ID No. 53; SEQ ID No. 54; SEQ ID No. 55; SEQ ID No. 56; SEQ ID No. 57; SEQ ID No. 58; SEQ ID No. 59; SEQ ID No. 60; SEQ ID No. 61; SEQ ID No. 62; SEQ ID No. 63; SEQ ID No. 64; SEQ ID No. 65; SEQ ID No. 66; SEQ ID No. 67; SEQ ID No. 68; and SEQ ID No. 69.

11. The method of claim 2, wherein said HS 19 PCR primers recognize HS19 Penner serotype; HS 63 PCR primers recognize HS63 Penner serotype; HS33 PCR primers

recognize HS33 and HS35 Penner serotypes; HS57 PCR primers recognize HS57 Penner serotype; HS12 PCR primers recognize HS12 Penner serotype; HS27 PCR primers recognize HS27 Penner serotype; HS21 PCR primers recognize HS21 Penner serotype; HS31 PCR primers recognize HS31 Penner serotype; HS62 PCR primers recognize HS62 Penner serotype; HS62 PCR primers recognize HS62 Penner serotype; HS45 PCR primers recognize HS45 Penner serotype; HS29 PCR primers recognize HS29 Penner serotype; HS22 PCR primers recognize HS22 Penner serotype; HS9 PCR primers recognize HS9 Penner serotype; HS37 PCR primers recognize HS37 Penner serotype; HS18 PCR primers recognize HS18 Penner serotype; HS58 PCR primers recognize HS58 Penner serotype; HS52 PCR primers recognize HS52 Penner serotype; HS60 PCR primers recognize HS60 Penner serotype; HS55 PCR primers recognize HS55 Penner serotype; HS32 PCR primers recognize HS Penner serotype; HS11 PCR primers recognize HS11 Penner serotype; HS40 PCR primers recognize HS40 Penner serotype; and HS38 PCR primers recognize HS38 Penner serotype.

12. The method of claim 3, wherein the amplification of products are analyzed by agarose gel electrophoresis.

13. The method of claim 5, wherein said PCR primer pairs are grouped into an alpha mix; a beta mix; a gamma mix and a delta mix, wherein each of said mixes comprise PCR primer pairs so that each PCR product within a mix differs by at least 20 bp.

14. A kit for typing *Campylobacter jejuni* strains, wherein the kit comprises one or more PCR primer pairs of claim 1.

15. The kit of claim 14, wherein said polysaccharide capsule loci is derived from *Campylobacter jejuni* strains selected from the strains: HS19, HS63, HS33, HS35, HS57,

HS12, HS27, HS21, HS31, HS62, HS45, HS29, HS22, HS9, HS37, HS18, HS58, HS52, HS60, HS55, HS32, HS11, HS40, and HS38.

16. The kit of claim 14, wherein said PCR primer pairs contain sequences selected from the group consisting of: SEQ ID No. 1 and SEQ ID No. 2; SEQ ID No. 3 and SEQ ID No. 4; SEQ ID No. 5 and SEQ ID No. 6; SEQ ID No. 7 and SEQ ID No. 8; SEQ ID No. 9 and SEQ ID No. 10; SEQ ID No. 11 and SEQ ID No. 12; SEQ ID No. 13 and SEQ ID No. 14; SEQ ID No. 15 and SEQ ID No. 16; SEQ ID No. 17 and SEQ ID No. 18; SEQ ID No. 19 and SEQ ID No. 20; SEQ ID No. 21 and SEQ ID No. 22; SEQ ID No. 23 and SEQ ID No. 24; SEQ ID No. 25 and SEQ ID No. 26; SEQ ID No. 27 and SEQ ID No. 28; SEQ ID No.29 and SEQ ID No. 30; SEQ ID No. 31 and SEQ ID No. 32; SEQ ID No. 33 and SEQ ID No. 34; SEQ ID No. 35 and SEQ ID No. 36; SEQ ID No. 37 and SEQ ID No. 38; SEQ ID No. 39 and SEQ ID No. 40; SEQ ID No. 41 and SEQ ID No. 42; SEQ ID No. 43 and SEQ ID No. 44; and SEQ ID No. 45 and SEQ ID No. 46.

17. The kit of claim 14, wherein said PCR primer pairs are grouped into an alpha mix comprising one or more sequence pairs selected from the group consisting of: SEQ ID No: 1 and SEQ ID No: 2; SEQ ID No: 3 and SEQ ID No: 4; SEQ ID No: 5 and SEQ ID No: 6; a beta mix comprising one or more sequence pairs selected from the group consisting of: SEQ ID No: 7 and SEQ ID No: 8; SEQ ID No: 9 and SEQ ID No: 10; SEQ ID No: 11 and SEQ ID No: 12; SEQ ID No: 13 and SEQ ID No: 14; SEQ ID No: 15 and SEQ ID No: 16; a gamma mix comprising one or more sequence pairs selected from the group consisting of: SEQ ID No: 17 and SEQ ID No: 18; SEQ ID No: 19 and SEQ ID No: 20; SEQ ID No: 21 and SEQ ID No: 22; SEQ ID No: 23 and SEQ ID No: 24; SEQ ID No: 25 and SEQ ID No: 26; SEQ ID No 27 and SEQ ID No: 28; and SEQ ID

No. 29 and SEQ ID No. 30; and a delta mix comprising one or more sequence pairs selected from the group consisting of: SEQ ID No. 31 and SEQ ID No. 32; SEQ ID No. 33 and SEQ ID No. 34; SEQ ID No. 35 and SEQ ID No. 36; SEQ ID No. 37and SEQ ID No. 38; SEQ ID No.39 and SEQ ID No. 40; SEQ ID No. 41 and SEQ ID No. 42; SEQ ID No. 43 and SEQ ID No.44; and SEQ ID No. 45 and SEQ ID No. 46.

18. The kit of claim 14, further comprising a buffer, diluents and/or excipient.

19. The kit of claim 14, further comprising a DNA polymerase.

20. The kit of claim 14, wherein the primers have about 18-30 nucleotides, a G/C content of 20-50%, and a melting temperature between about 57°C and 63°C.

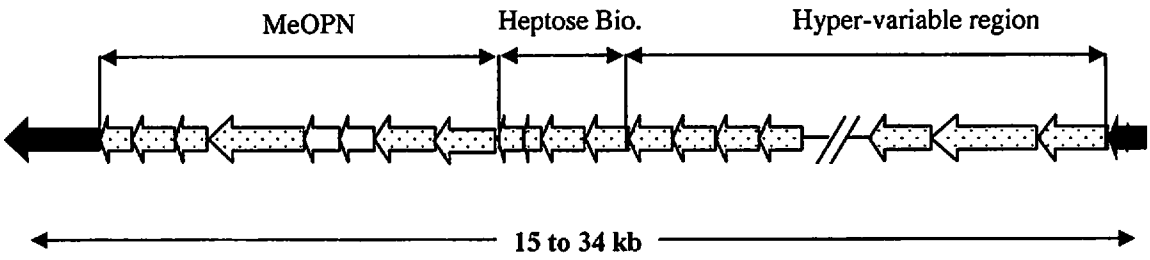

FIG 1

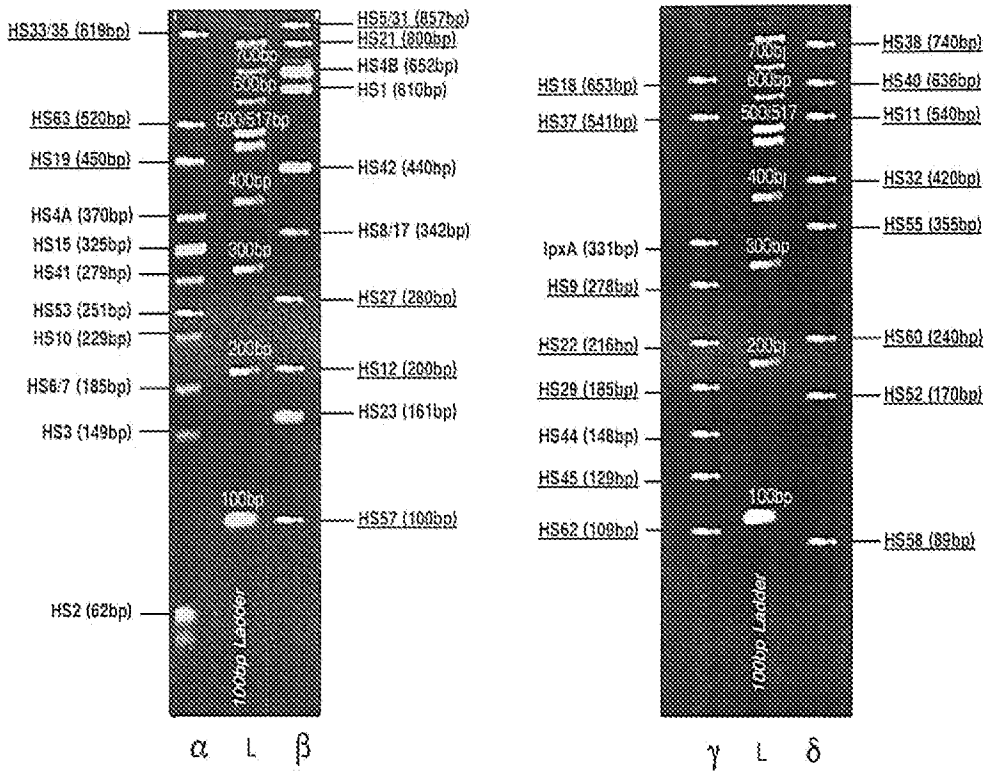
HS33/35 (618bp)
HS63 (520bp)
HS19 (450bp)
HS4A (370bp)
HS15 (325bp)
HS41 (279bp)
HS53 (251bp)
HS10 (229bp)
HS6/7 (185bp)
HS3 (149bp)
HS2 (62bp)
100bp Ladder
HS5/31 (857bp)
HS21 (809bp)
HS48 (652bp)
HS1 (610bp)
HS42 (440bp)
HS8/17 (342bp)
HS27 (280bp)
HS12 (200bp)
HS23 (161bp)
HS57 (100bp)
α L β
PCR Primer mix (group)
(L = mw ladder)
HS18 (653bp)
HS37 (541bp)
lpxA (331bp)
HS9 (278bp)
HS22 (216bp)
HS29 (185bp)
HS44 (148bp)
HS45 (129bp)
HS62 (109bp)
100bp Ladder
HS38 (740bp)
HS40 (636bp)
HS11 (540bp)
HS32 (420bp)
HS55 (355bp)
HS60 (240bp)
HS52 (170bp)
HS58 (89bp)
γ L δ
PCR Primer mix (group)
(L = mw ladder)

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/54309

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68; C12P 19/34; C07H 21/04 (2014.01)
USPC - 435/6.15, 91.2; 536/24.33
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) Classification(s): C12Q 1/68, 1/04, 1/00; C12P 19/34, 19/30, 19/28, 19/26, 19/00; C07H 21/04, 21/00 (2014.01)
USPC Classification(s): 435/6.15, 6.12, 6.1, 4, 91.2, 91.1, 89, 85, 84, 72, 41; 536/24.32, 24.33, 24.3, 23.1, 22.1, 18.7, 1.11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google/Google Scholar; Pubmed/Pubmed central/NCBI Blast; UniProt; 'Campylobacter jejuni', 'polysaccharide capsule', 'bacterial capsule', PCR, 'Polymerase chain reaction', 'hypervariable region', HVR, loci, locus, 'O-methyl phosphoramidate', MeOPN, 'Heptose synthesis', HS19

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2011/0207138 A1 (POLY, F et al.) August 25, 2011; paragraphs [0011]-[0049]; Claims 1- 20	1, 3, 5-9, 12-14, 18-20 -- 2, 4, 10, 11, 15, 16
Y	KARLYSHEV, A et al. Analysis of Campylobacter jejuni Capsular Loci Reveals Multiple Mechanisms for the Generation of Structural Diversity and the Ability to Form Complex Heptoses. Molecular Microbiology. January 2005, Vol. 55, No. 1, pp. 90–103; including GenBank accession BX545860; page 91, second column, second paragraph; page 92, table 1; page 92, first column, second paragraph to second column, first paragraph; page 100, second column, third paragraph; GenBank supplement, pages 1-9	2, 4, 10, 11, 15, 16
A	US 5494795 A (GUERRY, P et al.) February 27, 1996; entire document	1-16, 18-20
A	US 6355435 B1 (WILSON, D et al.) March 12, 2002; entire document	1-16, 18-20
A	US 2003/0113757 A1 (CZAJKA, J) June 19, 2003; entire document	1-16, 18-20
A	US 2007/0134652 A1 (SLEPNEV, V et al.) June 14, 2007; entire document	1-16, 18-20

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 February 2014 (21.02.2014)	07 MAR 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/54309

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/54309

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
-***-Please See Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Groups I+: Claims 1-16, 18-20, SEQ ID NOs: 1 (PCR primer of a primer pair nucleic acid sequence), 2 (PCR primer of a primer pair nucleic acid sequence), 47 (Campylobacter jejuni amplification product DNA nucleic acid sequence)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/54309

-***-Continued from Box III: Observations where unity of invention is lacking -***-

-Note: Regarding Claim 11, applicant is entitled to first named invention and thus for the purposes of this instant PCT application examination, said HS 19 PCR primers that recognize HS19 Penner serotype will be searched.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+: Claims 1-16 and 18-20, and SEQ ID NOs: 1 (PCR primer of a primer pair nucleic acid sequence), 2 (PCR primer of a primer pair nucleic acid sequence) and 47 (campylobacter jejuni amplification product DNA nucleic acid sequence) are directed toward a method of identifying Campylobacter jejuni strains in a sample suspected of containing Campylobacter jejuni DNA by polymerase chain reaction, wherein the amplification products of said polymerase chain reaction are derived from genes within the Campylobacter jejuni polysaccharide capsule (CPS) loci, comprising: (a) subjecting DNA from said sample to a PCR amplification reaction using one or more PCR primer pairs targeting one or more regions of the C.jejuni O-methyl phosphoramidate synthesis region, heptose synthesis and hyper-variable region of the Campylobacter jejuni polysaccharide capsule loci; (b) analyzing amplification products resulting from said amplification reaction.

The method of identifying Campylobacter jejuni strains in a sample suspected of containing Campylobacter jejuni DNA by polymerase chain reaction will be searched to the extent that it encompasses SEQ ID NOs: 1 (PCR primer of a primer pair nucleic acid sequence), 2 (PCR primer of a primer pair nucleic acid sequence) and 47 (campylobacter jejuni amplification product DNA nucleic acid sequence). It is believed that claims 1-16 and 18-20 encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NOs: 1 (PCR primer of a primer pair nucleic acid sequence), 2 (PCR primer of a primer pair nucleic acid sequence) and 47 (campylobacter jejuni amplification product DNA nucleic acid sequence). Applicants must indicate, if applicable, the claims which encompass the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An Exemplary Election would be: SEQ ID NOs: 3 (PCR primer of a primer pair nucleic acid sequence) and 4 (PCR primer of a primer pair nucleic acid sequence).

Groups I+ share the technical features including a method of identifying Campylobacter jejuni strains in a sample suspected of containing Campylobacter jejuni DNA by polymerase chain reaction, wherein the amplification products of said polymerase chain reaction are derived from genes within the Campylobacter jejuni polysaccharide capsule (CPS) loci, comprising: (a) subjecting DNA from said sample to a PCR amplification reaction using one or more PCR primer pairs targeting one or more regions of the C. jejuni O-methyl phosphoramidate synthesis region, heptose synthesis and hyper-variable region of the Campylobacter jejuni polysaccharide capsule loci; (b) analyzing amplification products resulting from said amplification reaction.

However, these shared technical features are previously disclosed by US 2011/0207138 A1 to Poly, et al. (hereinafter 'Poly'). Poly discloses a method of identifying Campylobacter jejuni strains (a method of identifying Campylobacter jejuni strains; abstract, Claim 1) in a sample suspected of containing Campylobacter jejuni DNA (in a sample suspected of containing Campylobacter jejuni; paragraph [0041], Claim 1) by polymerase chain reaction (by polymerase chain reaction; abstract; Claim 1), wherein the amplification products of said polymerase chain reaction (amplification products of the polymerase chain reaction; paragraph [0036]) are derived from genes within the Campylobacter jejuni polysaccharide capsule (CPS) loci (derived from genes within the Campylobacter jejuni polysaccharide capsule (CPS) loci; paragraph [0035]), comprising: (a) subjecting DNA from said sample to a PCR amplification reaction (subjecting DNA from said sample to a PCR amplification reaction; paragraph [0043], Claim 1) using one or more PCR primer pairs (one or more primer pairs; paragraph [0042], Claim 1) targeting one or more regions of the C. jejuni O-methyl phosphoramidate synthesis region (targeting one or more regions of the C. jejuni O-methyl phosphoramidate synthesis region; paragraph [0035], Claim 1), heptose synthesis and hyper-variable region of the Campylobacter jejuni polysaccharide capsule loci (heptose synthesis and hyper-variable region of the Campylobacter jejuni polysaccharide capsule loci; Claim 1) ; (b) analyzing amplification products resulting from said amplification reaction (analyzing amplification products resulting from said amplification reaction; Claim 1).

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Poly reference, unity of invention is lacking.