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Characterisation of chicken *Campylobacter jejuni* isolates using resolution optimised single nucleotide polymorphisms and binary gene markers

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ABSTRACT

The principal objective of this study was to determine if *Campylobacter jejuni* genotyping methods based upon resolution optimised sets of single nucleotide polymorphisms (SNPs) and binary genetic markers were capable of identifying epidemiologically linked clusters of chicken-derived isolates. Eighty-eight *C. jejuni* isolates of known *flaA* RFLP type were included in the study. They encompassed three groups of ten isolates that were obtained at the same time and place and possessed the same *flaA* type. These were regarded as being epidemiologically linked. Twenty-six unlinked *C. jejuni flaA* type I isolates were included to test the ability of SNP and binary typing to resolve isolates that were not resolved by *flaA* RFLP. The remaining isolates were of different *flaA* types. All isolates were typed by real-time PCR interrogation of the resolution optimised sets of SNPs and binary markers. According to each typing method, the three epidemiologically linked clusters were three different clones that were well resolved from the other isolates. The 26 unlinked *C. jejuni flaA* type I isolates were resolved into 14 SNP-binary types, indicating that *flaA* typing can be unreliable for revealing epidemiological linkage. Comparison of the data with data from a fully typed set of isolates associated with human infection revealed that abundant lineages in the chicken isolates that were also found in the human isolates belonged to clonal complex (CC) -21 and CC-353, with the usually rare C-353 member ST-524 being especially abundant in the chicken collection. The chicken isolates selected to be diverse according to *flaA* were also diverse according to SNP and binary typing. It was observed that CC-48 was absent in the chicken isolates, despite being very common in Australian human infection isolates, indicating that this may be a major cause of human disease that is not chicken associated.

1. Introduction

Infections caused by campylobacters are a major cause of food-borne illnesses in Australia and most other developed countries, with *Campylobacter jejuni* causing the great majority of human disease (Mickan et al., 2007; Humphrey et al., 2007; Moore et al., 2005). Unlike other agents of bacterial food-borne disease such as *Salmonella enterica*, *Campylobacter* spp. infections are largely sporadic rather than occurring in outbreaks (Snelling et al., 2005), making it difficult to understand the population biology and the relationships between genotype and virulence for these species, and to fully elucidate the transmission events that result in human disease.

An important tool in the understanding and minimisation of transmission of *Campylobacter* disease to humans is genetic fingerprinting (genotyping). One well accepted genotyping method is pulsed-field gel electrophoresis (PFGE). This is currently regarded as a gold standard because of its high resolving power, but it is also time consuming to perform and exacting to standardise between different laboratories (Gerner-Smidt et al., 2006). Other commonly used methods are based upon either extensive sequencing e.g. multilocus sequence typing (MLST) (Dingle et al., 2001), the characterisation of single hyper-variable loci such as *flaA* (Meinersmann et al., 1997), CRISPR (Schouls et al., 2003), and *cmp* (Huang et al., 2005), or the generation and electrophoretic separation of PCR products (e.g. amplified fragment length polymorphism (AFLP) analysis) (Schouls et al., 2003). Arguably, none of these techniques achieves an ideal combination of simplicity, convenience, and an adequate degree of resolving power to reliably detect epidemiological linkage.

Our research group has pursued the development of bacterial genotyping methods based upon real-time PCR technology. The advantages of the real-time PCR platform are that it is rapid, single step and closed tube, different classes of genetic polymorphisms can be interrogated, and the results are readily digitised (Barken et al., 2007; Mackay, 2007). An important aspect of our approach to the

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design of genotyping methods is the computer-aided derivation of resolution optimised sets of genetic markers from databases of comparative genetic information, which is carried out using the computer program "Minimum SNPs" (Robertson et al., 2004) "Minimum SNPs" takes large sequence alignments (e.g. entire MLST databases) as input, and provides as output sets of single nucleotide polymorphisms (SNPs) that are optimised with respect to Simpson's Index of Diversity (*D*) calculated against the input alignment. This approach has previously been applied to *Campylobacter* spp. (Price et al., 2006a). A set of seven *D*-optimised SNPs was derived from the combined *C. jejuni*/*C. coli* MLST database. An allele specific real-time PCR method approach to interrogating these SNPs was developed, and shown to provide a high degree of resolution against a collection of *C. jejuni* and *C. coli* isolates from human disease (Price et al., 2006a). Another set of markers for genotyping these species was derived from microarray-based comparative genome hybridisation (CGH) data (Price et al., 2006b). In this case, gene presence/absence data was converted into a pseudo-DNA sequence that could be analysed using "Minimum SNPs", and a set of eight *D*-optimised binary markers was identified. A real-time PCR method for interrogating these markers

Table 1
List of isolates indicating source, epidemiological origin and genotyping results using *flaA* RFLP, real-time PCR-based SNP* and binary gene* detection

PCR code	Study code	Source	*flaA* types	SNP profile[a]	Binary profile[b]	SNP+binary types
L1	Cycle 2, Shed 1, Day 36	Chicken faeces	XXVI	ACA1AGCC	IAPAPAAA	SNPT-1, BT-1
L2	Cycle 2, Shed 3, Day 37	Chicken faeces	I	GCA1AGCC	PAPAPAAA	SNPT-2, BT-30
L3	Cycle 2, Shed 6, Day 28	Chicken faeces	VIII	ACGGACT1	PPPPAAP	SNPT-5, BT-21
C354	#15,2,2	Chicken faeces	I	GCA1AG CC	PAPAPAAA	SNPT-2, BT-30
C488	#31,2,3	Chicken faeces	I	GCA1AGCC	PAPAPAAA	SNPT-2, BT-30
C495	#32, 13, 2L	Chicken faeces	I	GCA1AGCC	PAPAPAAA	SNPT-2, BT-30
C586	#43,1,5	Chicken faeces	I	GCA1AGCC	PAPAPAAA	SNPT-2, BT-30
C612	#49,3,5	Chicken faeces	I	GCA1AGCC	PAPAPAAA	SNPT-2, BT-30
C916	B1, 68, Day 31	Chicken faeces	I	GCA1AGCC	PAPAPAAA	SNPT-2, BT-30
H25	Cycle 2, Shed 2, Day 44	Chicken faeces	I	GCA1AGCC	PAPAPAAA	SNPT-2, BT-30
J185	Cycle 2, Shed 5, Day 50	Chicken faeces	I	GCA1AGCC	PAPAPAAA	SNPT-2, BT-30
C838	A1, 4, Day 42	Chicken faeces	I	GCA1AGCC	AAPPPAAP	SNPT-2,T-16
C734	#11A,3,1	Chicken faeces	I	ACA1GACT1	PAPPPPPP	SNPT-4, BT-8
M2	Cycle 1, Shed 4, Day 45	Chicken faeces	I	ACGGACT1	PAPPPAAP	SNPT-5, BT-14
C656	#54,1,2	Chicken faeces	I	ACGGACT1	PPPPPAAP	SNPT-5, BT-21
L176	Cycle 2, Shed 3, Day 49	Chicken faeces	I	ACGGACT1	PPPPPAAP	SNPT-5, BT-21
C607	#45,3,6R	Chicken faeces	I	ACGGACT1	PPPPPAAP	SNPT-5, BT-21
C729	#57,1,5	Chicken faeces	I	ACGGACT1	PPPPPAAP	SNPT-5, BT-21
G141	Cycle 2, Shed 3, Day 49	Chicken faeces	I	ACGGACT1	PPPPPPAP	SNPT-5, BT-25
C1267	N1, 3, Day 45	Chicken faeces	I	ACGGACT1	PPPPPPAP	SNPT-5, BT25
K35	Cycle 1, Shed 1, Day 51	Chicken faeces	I	ACGGACT1	IPPPPPPP	SNPT-5, BT-50
DAQ282A-909	#91, C3, Day 46	Chicken faeces	I	ACGGACC	PPAPPAAP	SNPT-11, BT-38
DAQ282A-263	#34	Crate	I	GCGGACT1	PPPPPAAP	SNPT-23, BT-21
C475	#28,83,6	Chicken faeces	I	AT2A1AACC	PPPPPAPP	SNPT-30, BT-26
P2	Cycle 1, Shed 2, Day 36	Chicken faeces	I	ACGAACT1	PPPPPAAP	SNPT-34, BT-21
C1214	F1, 2, Day 49	Chicken faeces	I	AT1A1AATC	AAPAPAAP	SNPT-35, BT-48
C628	#51,35,2S	Chicken faeces	I	GT1A1AGCC	PAPAPAAA	SNPT-36, BT-30
DAQ282A-1123	#64, C1, Day 27	Chicken faeces	I	GT1A1AACT1	PPPPPAPP	SNPT-37, BT-26
DAQ282A-190	#89	Crate	I	GT1A1AACT1	PIPPPAPP	SNPT-37, BT-39
N15	Cycle 1, Shed 1, Day 47	Chicken faeces	LIII (XXIV)	GT1A1GGCC	AAAAPAAA	SNPT-6, BT-17
C1266	L1, 1, Day 49	Chicken faeces	XVII	GT1A1GGTC	APAAAAAA	SNPT-6, BT-45
C1282	L2, 1, Day 46	Chicken faeces	XXII	GT1A1GGTC	AIAPPAAA	SNPT-6, BT-46
C654	#9A,6,6a	Chicken faeces	X	ACGGACC	AAPPPAAP	SNPT-11, BT-16
C858	A2, BJ9, 1	Chicken faeces	VIII	ACGGACC	PAPAPAAA	SNPT-11, BT-30
C1212	E1, 2, Day 35	Chicken faeces	V	GCGGACC	PAPPPAAP	SNPT-12, BT-14
C1275	B1	Crate	XXVII	GCGGACC	AAAPPAAI	SNPT-12, BT-49
C1273	BJ11, Shed 1	Chicken faeces	XXIV	GT1GAACT2	PAPPAAAA	SNPT-13, BT-41
C660	#54,1,10	Dropping	XI	GCGAATC	AAPPAAAP	SNPT-14, BT-15
DAQ282A-898	#91, C3, Day 46	Dropping	LXI	GCGAACC	AAPPAAAP	SNPT-15, BT-15
C338	#14,1,10	Dropping	XIV	GCGAACC	AAPPPAAP	SNPT-15, BT-16
C1271*	BJ10, Shed 3	Dropping	XXI	AT1A1GACC	AAPAPAAA	SNPT-18, BT-31
C541	#33,1,7	Dropping	XVI	ACA1GATC	AAPAPAAA	SNPT-21, BT-31
C1270	BJ10, Shed 2	Dropping	XXIII	AT1GGACC	AAAAPAAA	SNPT-22, BT-17
DAQ282A-921	#91, C3, Day 46	Dropping	LVI	AT1GGACC	AAPAPAAA	SNPT-22, BT-31
C627	#51,35,1	Dropping	XII	AT1GGACC	PPPPAAPP	SNPT-22, BT-37
C1209	A2, 5, Day 31	Chicken faeces	III	ACA1GACC	IAPPPAAP	SNPT-28, BT-42
C1272	BJ10, Shed 4	Chicken faeces	XIX	ACA1GACC	IAPIAAAP	SNPT-30, BT-43
C1211	Ballam Hill	Chicken faeces	IV	AT1GAACC	AAAAPAAA	SNPT-29, BT-17
C350	#15,1,3	Chicken faeces	XV	AT1GAACC	IAAAPAAP	SNPT-29, BT-44
C576	#40,5,7	Chicken faeces	XIII	AT1A1AATT1	IAPPPAPP	SNPT-31, BT-7
DAQ282A-639	#91, C2, Day 39	Chicken faeces	XLVII	GT1GAGCC	APAAPAAA	SNPT-32, BT-20
DAQ282A-698	#91, C2, Day 38	Larvae	XLVIII	ACGAGCC	AAAAPAAA	SNPT-33, BT-17
C1269	BJ10, Shed 2	Chicken faeces	XVIII	AT1A1AATC	AAPPPAAA	SNPT-35, BT-18
C1210	A3, 2, Day 39	Chicken faeces	VI	GT1GAATC	AIAAAAAA	SNPT-38, BT-47

*Protocol used as described in Price et al., 2006a.
L1, L2 and L3 are the three groups of epidemiologically linked isolates and the PCR codes of the isolates are listed below:
L1 — N70, N72, N73, N74, N75, N78, N79, N81, N82, N84.
L2 — A529, A531, A533, A535, A537, C1077, C1078, C1079, C1080 and C1081.
L3 — L131, L132, L133, L134, L136, L137, L138, L140, L141, L142.
Binary gene status P, I and A stand for Present, Intermediate and Absent.
[a] MLST-SNP profiles in the order of *aspA*174, *glnA*369, *gltA*12, *glyA*267, *pgm*348, *tkt*297 and *uncA*189.
[b] Binary gene profiles in the order of *Cj0629*, *Cj0265c*, *Cj0178*, *Cj0299*, *Cj1317*, *Cj1723c*, *Cj0008* and *Cj0486*.