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RESEARCH ARTICLE

Variability of Point Mutation Rates in *Campylobacter coli*: Preliminary Data and Observations

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ABSTRACT

In this study we evaluated the *in vitro* variability of *flaA* in *Campylobacter coli* strains (Co19 and Co29) isolated from distinct poultry farms. Strains were characterized by *flaA* PCR-RFLP and subcultured in absence or presence of *Salmonella enterica* (*S. Gallinarum*) as a host competitor in poultry. Only transition mutations were detected and no *flaA-flaB* recombination events were observed. The highest ratio of *flaA* mutations was detected in the presence of competitor ($1x10^{-6}$ and $4.8x10^{-6}$ for Co19 and Co29, respectively). While, transitions in absence of competitor were observed only in the strain Co29 ($5.8x10^{-7}$). Data obtained in this investigation represent a preliminary study aimed to evaluate the possible linkage between specific haplotypes and their proneness to acquire point mutations which in turn might help strains to gain a better fitness for survival within and outside hosts.

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INTRODUCTION

Campylobacter species, in particular C. *jejuni* and C. *coli,* are the major causes of human bacterial gastroenteritis worldwide (Cody *et al.*, 2013) During 2009, 198,256 human cases were notified in Europe, overtaking the notified cases for human salmonellosis (European Food Safety Authority and European Centre for Disease Prevention and Control, 2011).

The consumption of chicken meat represents one of the most important routes for human infection as livestock, and poultry, in particular, has been widely recognized as a primary reservoir for *C. jejuni* and *C. coli* (Elvers *et al.*, 2011). However, *Campylobacter* spp. are also able to survive *ex vivo* in cold and moist environments for long periods (Wilson *et al.*, 2008). Some Authors hypothesized that the capability of *Campylobacter* spp. to survive and colonize dynamic environments such as the guts of different hosts may be associated with genetic variability. In particular, mechanisms such as natural competence, slip-strand mutations and genomic rearrangements could generate variegated phenotypes, which might show a better fitness to overcome environmental bottlenecks (Ridley *et al.*, 2008).

The colonization of both human and avian intestine by *Campylobacter* spp. requires the expression of a number of factors which control motility, adhesion to the intestine wall, cellular invasion and toxin production (Vandamme *et al.*, 2005; Wai *et al.*, 2012). The full sequencing of C. *jejuni* genome (strain NCTC 11168) has shown the presence of hypervariable regions, some of which are associated to genes involved in the synthesis of bacterial surface elements, such as lipooligosaccharides, surface polysaccharides and flagella (Parkhill *et al.*, 2000). In general, the variation of bacterial surface elements might represent a possible way to evade the host immune system. Among these elements, flagella play a key role during the processes of host colonization and cellular invasion. (Dasti *et al.*, 2010). The flagellin of *C. jejuni* and *C. coli* is encoded by the genes *fla*A and *fla*B. These genes share 95% sequence identity, they are arranged in tandem and separated by approximately 200 nucleotides (Harrington *et al.*, 1997). Their expression is also differently regulated. While *fla*A appears to be critical for motility, colonization, and pathogenesis, *fla*B represents a non-functional reservoir for genetic diversity. Recombination between *fla*A and *fla*B increases variability of the former and then the ability to better survive and/or colonize other hosts (Ridley *et al.*, 2008). The analysis of *fla*A has then been used for strains typing within the species of C. *jejuni* and C. *coli* (Merchant-Patel *et al.*, 2010). Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) is currently one of the most discriminatory techniques employed for analysis of *flaA*. Indeed, PCR-RFLP is simple, affordable, and profiles generated by this technique may be easily

compared with those already published (Eberle and Kiess, 2012).

It should be underlined that *flaA* PCR-RFLP does not discriminate between *C. jejuni* and *C. coli* (Behringer *et al*., 2011). However, *flaA* PCR-RFLP is particularly useful to track down the *flaA* gene distribution and its evolution among the isolates of these species. The present study was aimed to evaluate the extension of variability of *flaA* in *C. coli*, and if such variability were linked to specific *flaA* sequence types and/or to exposure at competitive stress conditions.

MATERIALS AND METHODS

Sampling and bacterial strains: Sixty cloacal samples were collected from two laying hen flocks (A and B) of two distinct poultry farms. The farms were located in Apulia, Southern Italy, and the samplings were performed on 2010, during January (flock A) and March (flock B). Birds were about 35 week old, at the best of their egg production stage.

All samples were collected by using sterile swabs and Cary-Blair (Copan, Brescia, Italy) as transport medium and processed within 3 hours from collection. The cultures from swabs were carried out according to the method 5 described by Acke *et al.* (2009). The identification of the isolates was performed by multiplex PCR according to Denis *et al.* (1999).

***In vitro* subculturing:** Two *C. coli* strains with different PCR-RFLP profiles and from different poultry farms were chosen and passaged in tryptic soy agar (Oxoid, Milan, Italy) plates supplemented with 5% defibrinated sheep blood (BTSA) up to 50 times or until viability appeared remarkably impaired.

Both strains were also subcultured on BTSA including 10^6 colony forming units (CFU) of *Salmonella enterica* subsp. *enterica* ser. Gallinarum (*S.* Gallinarum). Such species has been chosen because of its high host specificity. To avoid overwhelming growth of *S.* Gallinarum, each two passage the strains were passaged on BTSA with addition of 32 mg/L cefoperazone and 10 mg/L amphotericin B (Oxoid). In both cases, a single colony was passaged each time, as described by Leblanc-Maridor *et al.* (2011).

DNA extraction and PCR-RFLP: For PCR-RFLP, three well distinct colonies were picked up for each subcultured strain at the first passage, while 10 colonies were picked up from each next passage. DNA was extracted by boiling the selected colonies and centrifuging the suspension. The supernatant was used as a template in the *flaA* PCR, performed according to Nachamkin *et al.* (1993) Digested fragments were separated by electrophoresis and the images of the ethidium bromide stained gels were digitalized. The molecular weights of the restriction fragments were determined and the *flaA* PCR-RFLP profiles were compared with those from literature. The determination of molecular weights was then confirmed by recognizing the *DdeI* restriction sites onto the *flaA* amplicon nucleotide sequences.

FlaA sequence analysis: For each strain, an aliquot of the PCR products from the first and the last passages, with and without *S.* Gallinarum, were purified and cloned by using the Zero Blunt® Cloning Kit (Life Technologies, Milan, Italy) according to the manufacturer's instruction. Twelve clones for each experiment were sequenced by using the Big Dye Terminator v3.1 (Applied Biosystems, Monza, Italy). The nucleotide sequences were compared by the Mega 5 software (Tamura *et al.*, 2011).

RESULTS

Thirteen isolates resulted positive for *C. coli* or *C. jejuni* out of the 60 collected samples (table 1). Three swabs, collected from the flock A, were positive for *C. jejuni*, 3 were positive for *C. coli* and 2 positive for both species. In the latter cases, we could not isolate the two species separately as the cultures gave confluent colonies. Therefore, we could not proceed with the subsequent genotyping. From flock B, 5 swabs were found positive for *C. jejuni* and 2 for *C. coli.* We did not retrieve mixed cultures from flock B.

Fla typing results: Two out of the 3 *C. jejuni* strains from the flock A exhibited the same PCR-RFLP profile (J2), while the third isolate was not typeable. Two strains from the flock B exhibited the profile J1, one J2 and one J4 (Fig. 1). The fifth isolate was not typeable. The non typeability of some strains was probably due to cross colonization of the same host by different strains.

The *C. coli* isolates only showed the profiles C1 and C2 (Fig. 2). The latter characterized an isolate from the flock B, while the C1 was exhibited by one isolate from the flock A and one from the flock B.

While distinct *C. jejuni* profiles were identified in flocks A and B, the C1 profile identified in *C. coli* was detected in both flocks. We then focused our attention on the common C1 profile for further analysis. The profile C2 was also included for comparison.

Additionally, the choice of strains with profiles C1 and C2 had also an epidemiological relevance in that we found that C2 characterized the majority of the *C. jejuni* and *C. coli* strains isolated in Eastern Turkey between 2000 and 2001 (Ertaş *et al.*, 2004). We also found that the profile C1 matched with those characterizing the strains CNET019, CNET020, CNET021 and CNET051 isolated in France and Netherlands (Harrington *et al.*, 2003). These profiles corresponded to the fla type 44 of the Campynet (CNET) database (accessible online at the address http://www.cbs.dtu.dk/cgi-bin/campynet/db.cgi?), which characterized a group of *C. jejuni* strains collected in Northern Europe.

Fla sequence analysis results: While the PCR-RFLP did not show any variation during and after subculturing, the comparison of *flaA* nucleotide sequences from the first and last passage highlighted some differences (Table 2). The nucleotide sequences of amplicons from the strain Co19, subcultured 43 times without competitors, did not vary at all. While, 2 synonymous substitutions (both T→C), were detected when the strain was subcultured in the presence of *S.* Gallinarum 43 times.

Table 1: Distribution and *flaA* PCR-RFLP characterization of *C. coli* and *C. jejuni* isolates in the sampled flocks

Flock (samples)	Isolates	*C. coli*	*C. jejuni*	*C. coli flaA* PCR-RFLP types (number of isolates)	*C. jejuni flaA* PCR-RFLP types (number of isolates)
A (20)	6	3	3	C1 (2), NT_M[a] (1)	J3 (2), NT[a] (1)
B (40)	7	2	5	C1 (1), C2 (1)	J1 (2), J2 (1), J4 (1), NT[b] (1)

[a]NT_M: not typeable because belonging to a mixed culture; [b]NT_T: not typeable due to technical reasons.

Table 2: Results of nucleotide sequence comparisons. Data are relative to the comparison of the sequences from last passage of subcultures with the sequences from parental strains.

Sequences[1]	Substitutions	Gaps	Transitions	Transversions	Non synonymous substitutions (amino acid mutation)
Co19(43)	0	0	0	0	0
Co19(50S)[2]	2	0	2 (T→C)	0	0
Co29(44)	1	0	1 (C→T)	0	1 (A→V)
Co29(30S)[2]	5	0	1 (G→A)	0	1 (E→G)
			2 (A→G)		
			2 (T→C)		

[1]Names of sequences specify the strain (Co19 or Co29) and the passages from which the sequence has been obtained (reported parenthetically). [2] *Campylobacter coli* grown in presence of *S.* Gallinarum

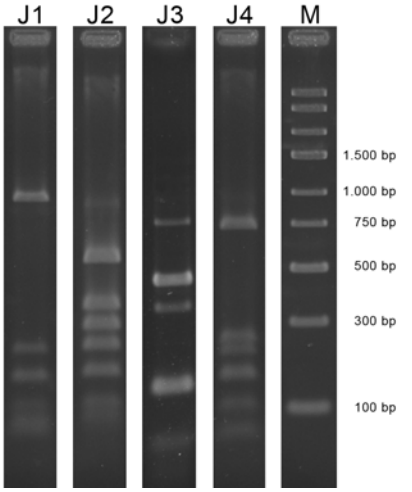

Fig. 1: *flaA* PCR-RFLP profiles of the isolated *Campylobacter jejuni* strains. J1-J4: PCR-RFLP profiles; M: O'GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific, Milan, Italy)

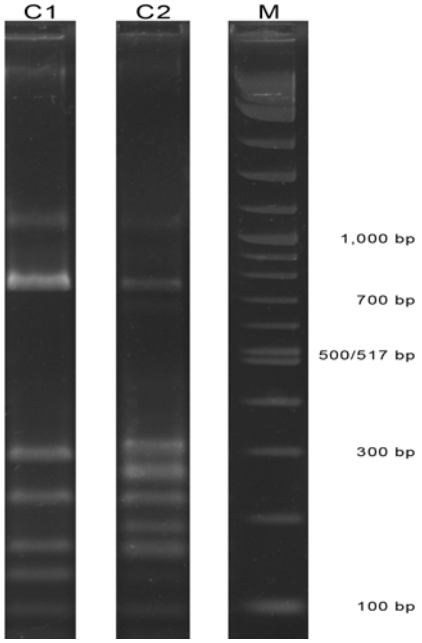

Fig. 2: *flaA* PCR-RFLP profiles of the isolated *Campylobacter coli* strains. C1-C2: PCR-RFLP profiles; M: 2-Log DNA Ladder (New England Biolabs, Ipswich, MA, US)

After 44 passages, the strain Co29 showed 1 non synonymous substitution (C→T) corresponding to the variation A→V at amino acid level. While the sequence from the same strain subcultured with the competitor exhibited 5 nucleotide variations after only 30 passages. Four of them were synonymous mutations; while one resulted in an E→G substitution at the amino acid level.

Assuming approximately that a colony includes about $5x10^5$ – 10^6 cells, and consequently that about 20 generations per passage were needed to form a colony, and considering the dimension of the analyzed fragment (1,725 and 1,722 bp for Co19 and Co29, respectively), we calculated a substitution rate between $5.8x10^{-7}$ (alone) and $4.8x10^{-6}$ (with competitor) for the strain Co29, and $1x10^{-6}$ for the strain Co19 in presence of the competitor. The *flaA* sequence of the strain Co19 seemed stable in absence of competitor, as no substitutions were retrieved after 43 passages. All the sequences of the same strain only differed by single nucleotide differences, without wider rearrangement ascribable to recombination events.

DISCUSSION

The low number of substitutions did not allow a careful statistical analysis, but our data highlighted that the *flaA* locus of the two analyzed strains had different mutation rates. In particular, *flaA* of Co19 remained unmodified even after 43 passages, while, in the same conditions, the approximate mutation rate of Co29 was $5.8x10^{-7}$. Previous data reported that the spontaneous mutation rates observed in the *gyrA* gene of *C. coli*, was 10^{-8}, which dropped to 10^{-3} in the hypermutable strains (Hanninen and Hannula, 2007). The mutation rates we observed with respect to *flaA* in *C. coli* were quite distant from values detected for *gyrA*, especially in presence of a competitor. In fact, when both strains were challenged with *S.* Gallinarum, the mutation rate showed a 1-order increase.

Ridley *et al*. (2008) underlined that competitive stress could induce genomic instability in *C. jejuni*. They also hypothesized a kind of host specific response to stress, and that variability may be linked to an increased probability for *C. jejuni* to colonize new hosts and/or to better survive to the environmental exposure which occurs during the host-to-host transmission

However, they found that recombination processes played a pivotal role in generating such variability and that these events did not involve *flaA* and *flaB*. Our data

confirmed such trend for *flaA* in *C. coli* too, but, on the other hand, they suggested a possible major role of the point mutation events. Furthermore, the observed substitutions were randomly scattered and not associated to hot spots, as repeats or G/C stretches which were found to increase the mutation rates (Bayliss *et al*., 2012). Our data suggests that *flaA* might have an intrinsic dynamicity, independent from the contribution mediated by recombination with *flaB*. Such dynamicity could also be modulated by exogenous stimuli such as competitiveness and nutritional stress. These factors would act by regulating, directly or indirectly, the mechanisms involved in generating point mutations.

In the light of this hypothesis it is reasonable to assume that different haplotypes of *C. coli* strains could be characterized by a different proneness to acquire point mutations. This could explain the low but not negligible differences in the point mutation rates which we observed in the two strains.

We observed only transitions and greater attention should be focused on those repair systems which are involved in recognition and repair of the altered structures which lead to purine→purine and pyrimidine→pyrimidine substitutions. Such systems, in *Escherichia coli*, involve *mutS* and *mutL* genes (Jiricny, 1998). However, *Campylobacter* spp. lacks both *mutL* and *mutS* homologs (Bayliss *et al.*, 2012). Identification and characterization of these repair systems in *Campylobacter* spp. might help to perform studies on the possible linkage between specific *C. coli* and *C. jejuni* haplotypes and their potential fitness to spread among their hosts (particularly avian and human). Such information could be useful also to assess the contribution of competitive stress to *Campylobacter* spp. variability.

It is also noteworthy that the most variable strain was Co29, whose *flaA* type was identified in strains from two different poultry farms. The same *flaA* type (namely the type 44 of CNET), is reported among *C. jejuni* strains isolated in Europe (Harrington *et al.*, 2003), and appeared widely spread. A further step should be the assessment of association, in *C. coli* and *C. jejuni* strains, among specific *fla*A types sequence variability and strains diffusion.

Conclusion: On aggregate, the presented results suggest that specific *C. coli* strains might be more prone than other to variability. Since genetic variability may help *Campylobacter* spp. during colonization of gut, it is tempting to speculate that specific haplotypes may grant a greater variability and, consequently, specific advantages, even in the presence of a competitor. Further studies should be addressed to verify this hypothesis.

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