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Multilocus sequence typing of *Campylobacter jejuni* and *Campylobacter coli* from humans and chickens in North-Eastern Italy

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SUMMARY

This paper reports the multilocus sequence typing (MLST) of 57 *C. jejuni* and *C. coli* isolates from humans and chickens in Italy and the identification of 17 new sequence types (STs). A high genetic diversity was detected among *C. jejuni*/*C. coli* and human/chicken isolates, with a predominance of clonal complexes CC21 and CC828. Although human STs were not the same as those found in chickens, 3 CCs overlapped between human and chicken isolates. Genotyping of *Campylobacter* strains by MLST should be encouraged in order to implement surveillance and control of infection in humans and in animal reservoirs in Italy.

KEY WORDS: Campylobacter jejuni, *Campylobacter coli*, MLST, Humans, Poultry, Zoonosis

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Thermotolerant campylobacters are among the leading bacterial pathogens involved in human gastroenteritis worldwide (Humphrey *et al.,* 2007). In the European Union (EU), the number of human campylobacteriosis cases has followed a significant increasing trend in the last years, with 220,209 confirmed cases in 2011 (EFSA, 2013). In Italy, 468 episodes of disease were documented in 2011 (0.77 confirmed cases per 100,000 population), showing a statistically significant increasing trend since 2008 (265 confirmed cases). However, it should be taken into account that campylobacteriosis is not a notifiable disease in Italy, thus it is considerably underreported (EFSA, 2013). In this regard, it has been estimated that the incidence of true cases should be 586 per year per 100,000 inhabitants/years (Havelaar *et al.,* 2013). The majority of human infections are sporadic and caused by *Campylobacter jejuni*, followed by *Campylobacter coli*. In some cases, gastrointestinal infection can progress to life-threatening extra-intestinal diseases (*i.e.* immunoproliferative small intestinal disease) or complications, such as reactive arthritis, Guillain-Barré and Miller-Fisher syndromes (EFSA, 2013).

C. jejuni and *C. coli* naturally colonize a wide range of domestic and wild animals (Humphrey *et al*., 2007). Poultry are recognized as the main reservoir of the microorganism and rarely show clinical signs of disease. In particular, chickens and turkeys carry *Campylobacter* at high concentrations in their intestinal tract, posing a high risk of carcass contamination during the slaughtering process. Handling and consumption of contaminated poultry meat is the major source of human campylobacteriosis. In the EU, it has been estimated that 50% to 80% of human cases may be attributed to the chicken

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reservoir as a whole, while the handling and consumption of broiler meat may account for 20% to 30% of cases (EFSA, 2013).

Molecular typing has been used in source attribution studies to estimate the relative contribution of different *Campylobacter* reservoirs and to investigate transmission routes for human campylobacteriosis (Wilson *et al.,* 2008; Ragimbeau *et al.,* 2009; Sheppard *et al.,* 2009a; Mughini Gras *et al.,* 2012; Kittl *et al.,* 2013). Currently, multilocus sequence typing (MLST) represents the method of choice for genotyping of *Campylobacter* spp. and it is extensively used for source attribution of human cases, as it assesses geographical, temporal and host species-related variation in type (Dingle *et al.,* 2005).

To our knowledge, MLST has not previously been used to genotype Italian *Campylobacter* isolates either in humans or in chickens. With the aim to fill this information gap, the present paper reports the first results regarding *C. jejuni* and *C. coli* genotypes implicated in human gastroenteritis cases in Italy and their genetic relatedness with chicken isolates.

Eighteen human *Campylobacter* strains (11 *C. jejuni* and 7 *C. coli*) and 39 chicken strains (23 *C. jejuni* and 16 *C. coli*) were included in the study. Chicken strains were selected to obtain a *C. jejuni*: *C. coli* ratio similar to human strains (59% and 41% for chickens and 61% and 39% for humans). Human strains were isolated from clinical cases of acute gastroenteritis during the routine activity of the Regional Reference Centre for Infectious Diseases in the Veneto region (North-Eastern Italy). Chicken isolates derived from a survey carried out in commercial farms located in North-Eastern Italy (Giacomelli *et al.,* 2012). Both human and chicken isolates were collected during 2009. Species identification was obtained by multiplex PCR, as previously reported (Giacomelli *et al.,* 2012).

MLST was performed according to the seven-loci schemes for *C. jejuni* and *C. coli*, employing the primer sets and experimental conditions suggested by the *Campylobacter* MLST database (http://pubmlst.org/campylobacter/). Genomic DNA was extracted from overnight cultures grown on tryptic soy agar (OXOID) supplemented with 5% horse blood (OXOID) by simple lysis of the bacterial cells. A half-loop of bacterial culture was suspended in 100 µl sterile RNase/DNase-free water (Sigma-Aldrich) and heat inactivated by boiling for 20 min. Cell lysates were stored at -20°C until use. PCR reactions were carried out in a final volume of 50 µl, each mixture including: 4 µg of *Campylobacter* DNA, 0.2 µM of forward and reverse primers, 1X PCR buffer, 2mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphates, and 1 U of High Fidelity Platinum Taq DNA polymerase (Invitrogen). PCR products were purified by using the High Pure PCR Cleanup Micro Kit (Roche Diagnostics) according to the manufacturer's instructions. Purified PCR products were sequenced by using the ABI PRISM BigDye® Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems) as indicated by the manufacturer, and analysed with the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Sequences were assembled by using the ChromasPro 1.42 (Technelysium Pty Ltd.) software and aligned to the alleles in the *C. jejuni* and *C. coli* PubMLST database to determine the allele numbers. The sequence type (ST) and the clonal complex (CC) of each allelic profile were designated by interrogation of the database. New allele sequences and STs were submitted to the curator of the *Campylobacter* PubMLST database for number designation.

Out of 57 *Campylobacter* strains, 35 different STs were identified: 16 (9 in *C. jejuni* and 7 in *C. coli*) in human strains and 19 (9 in *C. jejuni* and 10 in *C. coli*) in chicken strains (Table 1). Seventeen novel STs (6 in human and 11 in chicken isolates) and 2 new alleles (1 from humans and 1 from chickens) for the *glnA* (assigned no. 484) and *aspA* (assigned no. 356) loci, respectively, were detected in this study. Thirty-nine isolates grouped into 8 known CCs, while 19 isolates had STs not assigned to any CC (http://pubmlst.org/campylobacter/, last access date 31 January 2014) (Table 1). Among human and chicken isolates, 14 and 13 STs were grouped into 6 (5 for *C. jejuni* and 1 for *C. coli*) and 5 (3 for *C. jejuni* and 2 in *C. coli*) known CCs, respectively; while 2 and 6 STs from humans and chickens, respectively, did not belong to any CC (Figure 1). ST-828 CC and ST-21 CC predominated and accounted for 22.8% (n=13) and 21% (n=12) of all isolates, respectively (Figure 1). ST-828 CC, containing 11 different STs, was detected in 7 human and 6 chicken *C. coli* strains; ST-21 CC,

including 7 different STs, was identified in *C. jejuni* both from humans (1 isolate) and chickens (11 isolates). ST-21 CC, ST-443 CC and ST-828 CC were shared between human and chicken campylobacters, whereas no overlapping STs were found (Table 1).

This study is the first to provide data on MLST types and genetic relatedness between human and chicken *C. jejuni* and *C. coli* from Italy. Given the relatively small sample size and the limited time span considered in this study, an association between human and chicken campylobacters could not be assessed and therefore the role of chickens as source of human campylobacteriosis cases could not be established. Nevertheless, in view of the total absence of papers reporting MLST analyses from Italy, data generated in the present study should be considered valuable, since they account of human and chicken *Campylobacter* genotypes from a novel geographical location.

TABLE 1 - *C. jejuni and C. coli MLST genotypes among human and chicken isolates. New sequence types are shown in bold type.*

Source	*Campylobacter species*	*CC*	*ST*	*N.*
Human	*C. jejuni*	ST-257	257	2
			6852	1
		ST-206	572	2
		ST-443	443	1
			2361	1
		ST-21	**6851**	1
		ST-353	3327	1
		NA	**6850**	1
		NA	**6856**	1
	C. coli	ST-828	827	1
			829	1
			854	1
			1556	1
			2317	1
			6853	1
			6854	1
Chicken	*C. jejuni*	ST-21	50	6
			5553	1
			6862	1
			6863	1
			6864	1
			6865	1
		ST-446	2850	4
		ST-443	**6867**	1
		NA	3029	7
	C. coli	ST-828	2642	2
			6866	2
			3026	1
			6868	1
		ST-1150	**6877**	1
		NA	3031	3
		NA	5401	3
		NA	**6859**	1
		NA	**6860**	1
		NA	**6861**	1

NA = not assigned.

A high genetic diversity was detected among human and chicken *C. jejuni* and *C. coli* population examined herein, as widely documented all over the world (Dingle *et al.,* 2005; Sheppard *et al.,* 2009a; de Haan *et al.,* 2010; Griekspoor *et al*., 2010; Kittl *et al.,* 2013). Out of 57 *Campylobacter* isolates, MLST analysis revealed 18 known and 17 new STs, including 2 novel alleles (*glnA* and *aspA* in a human and in a chicken isolate, respectively). Twelve STs were assigned to previously defined CCs, five not. As reported by other authors (Kittl *et al.,* 2013), novel genotypes may represent local clones restricted to a given country. More detailed analyses are needed to assess this possibility for Italian strains. By comparing human and chicken *Campylobacter* isolates, we also noticed that a higher diversity in CCs was present among human *C. jejuni* compared to chicken isolates (5 different known CCs plus 2 unknown CCs *vs* 3 different CCs known CCs plus 1 unknown CC, respectively), which is consistent with previously published data (de Haan *et al*., 2010). On the other hand, a larger number of novel STs unassigned to a defined CC among chicken *C. coli* compared to human isolates was identified, in accordance with other studies (Manning *et al.,* 2003; Griekspoor *et al.,* 2010).

Results from our study support the observation that, despite the high genetic diversity usually detected within *Campylobacter* populations, a remarkable similarity among isolate collections on a national and international scale, even across different continents, can be documented (Colles and Maiden, 2012). Indeed, the CCs we identified among human *C. jejuni* isolates (*i.e.* ST-21 CC, ST-206 CC, ST-257 CC, ST-353 CC

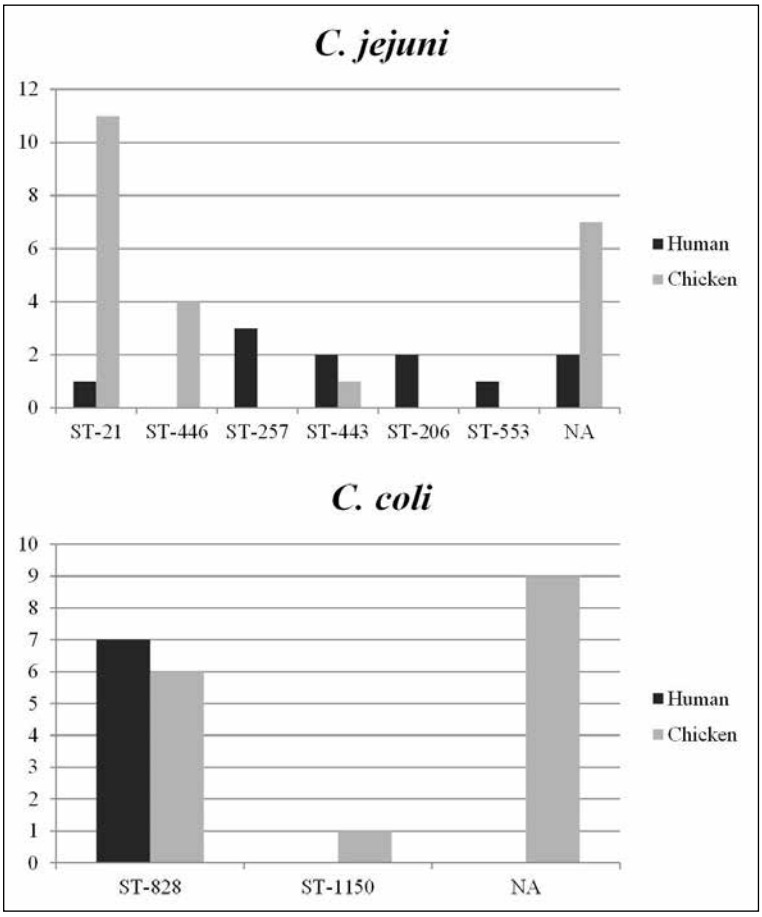

FIGURE 1 - *Distribution of human and chicken isolates in CCs. Not assigned CCs were pooled in the category "NA". Numbers on the y-axis indicate the number of isolates.*

and ST-443 CC) have been frequently associated with human disease and they are geographically widely distributed (Manning *et al.,* 2003; Ragimbeau *et al*., 2009; Sheppard *et al.,* 2009a; de Haan *et al.,* 2010; Colles and Maiden, 2012). Likewise, the most common CC among chicken *C. jejuni* isolates was the ST-21 CC according to that documented worldwide (Manning *et al.,* 2003; Ragimbeau *et al.,* 2009; Sheppard *et al.,* 2009b; Colles and Maiden, 2012; Kittl *et al.,* 2013). The other two CCs identified among chicken strains, the ST-443 CC and the ST-446 CC, have also been previously described (Manning *et al*., 2003; Ragimbeau *et al.,* 2009; Kittl *et al.,* 2013). Interestingly, in our study the ST-206 CC, the ST-257 CC and the ST-353 CC were detected only among human *C. jejuni*, whereas they have also been described in campylobacters from chickens (Manning *et al.,* 2003; Ragimbeau *et al.,* 2009; Sheppard *et al.,* 2009b). On the other hand, the ST-446 CC was detected only among chicken *C. jejuni*, but it has been described from cases of human campylobacteriosis in other European countries (Ragimbeau *et al.,* 2009). Regarding MLST types identified among human *C. coli* isolates, we found only one CC (*i.e.* ST-828 CC). Similarly, the most common CC detected in chicken isolates was the ST-828 CC. In addition, MLST analysis showed that the ST-1150 CC was present among chicken *C. coli*. The predominance of the ST-828 CC was expected since several studies demonstrated that this CC is globally spread in both human and chicken *C. coli* population (Sheppard *et al.,* 2009a; Colles and Maiden, 2012), whereas the ST-1150 CC has been rarely reported (Sheppard *et al.,* 2009b). In general, *C. jejuni* and *C. coli* genotypic population structure documented in the present study is in line with that previously reported. Indeed, *C. jejuni* populations are reported not strongly structured into differentiated clusters (Wilson *et al.,* 2008; Sheppard *et al.,*

2009a; Colles and Maiden, 2012), whereas *C. coli* population structure is limited and usually more restricted compared to *C. jejuni* (Dingle *et al.,* 2005; Colles and Maiden, 2012).

In the present study, no human STs were the same as those found in chickens, whereas three CCs (*i.e.* ST-21 CC, ST-443 CC and ST-828 CC) were found overlapping between human and chicken isolates. Of these, only three human *C. jejuni* isolates, *i.e.* one isolate (ST-21 CC) and two isolates (ST-443 CC), belonged to the same CC including also 11 and 1 isolates from chickens, respectively. Conversely, a more evident overlap between human and chicken *C. jejuni* genotypes has been reported by other authors (Ragimbeau *et al.,* 2009; Sheppard *et al*., 2009a; de Haan *et al.,* 2010; Kittl *et al*., 2013). It is noteworthy that ST-21 CC was predominant among chicken isolates, but rare among human isolates in our study. This CC is one of the most widely distributed among human and chicken *C. jejuni* populations worldwide and frequently overlapped between these two host species in several studies (Ragimbeau *et al.,* 2009; de Haan *et al.,* 2010; Kittl *et al.,* 2013). In general, this CC shows a large overlap in genetic variation among reservoirs, including both animal (*e.g.* cattle, sheep, pig, wild birds) and environmental sources (*e.g.* water, sand) (Manning *et al.,* 2003; Ragimbeau *et al*., 2009; Sheppard *et al.,* 2009b). Besides human and chicken campylobacters, ST-443 CC has also been isolated from other sources, such as the environment (Manning *et al., 2003*; Sheppard *et al.,* 2009b). In this study, all human *C. coli* isolates were included in the ST-828 CC, which was found prevalent also in chickens (7 out of 16 isolates). Other authors (Wilson *et al.,* 2008; Ragimbeau *et al.,* 2009; Sheppard *et al.,* 2009a; Kittl *et al.,* 2013) reported a similar overlap between human and chicken *C. coli*. This CC has been reported also in pigs, turkeys, cattle, sheep, and the environment (Sheppard *et al.,* 2009b; Griekspoor *et al.,* 2010).

Molecular typing has previously been used to infer the sources and routes of transmission of campylobacteriosis in humans. Overlapping of genotypes in *Campylobacter* species infecting humans and chickens suggested this food-producing animal as the main source of human infection (Wilson *et al.,* 2008; Ragimbeau *et al*., 2009; Sheppard *et al.,* 2009a; Kittl *et al.,* 2013). In our study, *Campylobacter* genotypes implicated in human gastroenteritis cases did not show a substantial match with isolates originating from chickens, suggesting a weak association between human and chicken *Campylobacter* isolates circulating in Italy. Human *Campylobacter* infection may indeed originate from other livestock and non-livestock sources, other than chickens (Manning *et al.,* 2003; Wilson *et al.,* 2008; Ragimbeau *et al.,* 2009; de Haan *et al.,* 2010; Colles and Maiden, 2012).

Campylobacter is a rapidly evolving species and investigation of the population structure and evolution is necessary to allow an efficient surveillance in humans and animals (Wilson *et al.,* 2008; Colles and Maiden, 2012). More data are essential to clarify genotype distribution and population structure of thermotolerant *Campylobacter* spp. and to identify potential sources of human campylobacteriosis in Italy. Genotyping of *Campylobacter* strains by multilocus sequence typing seems to be an essential tool for studying the epidemiology of *Campylobacter* and therefore should be encouraged to implement surveillance and control of infection in humans and in animal reservoirs in Italy.

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REFERENCES

Colles F.M., Maiden M.C.J. (2012). *Campylobacter* sequence typing: applications and future prospects. *Microbiology.* **158**, 2695-2709.

de Haan C.P., Kivisto R., Hakkinen M., Rautelin H., Hanninen M.L. (2010). Decreasing trend of overlapping multilocus sequence types between human and chicken *Campylobacter jejuni* isolates over a decade in Finland. *Appl. Environ. Microbiol.* **76**, 5228-5236.

Dingle K.E., Colles F.M., Falush D., Maiden M.C. (2005). Sequence typing and comparison of population biology of *Campylobacter coli* and *Campylobacter jejuni*. *J. Clin. Microbiol.* **43**, 340-347.

EFSA (European Food Safety Authority), ECDC (European Centre for Disease Prevention and Control). (2013). The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2011. *The EFSA Journal* **11,** 3196.

Giacomelli M., Andrighetto C., Rossi F., Lombardi A., Rizzotti L., Martini M., Piccirillo A. (2012). Molecular characterization and genotypic antimicrobial resistance analysis of *Campylobacter jejuni* and *Campylobacter coli* isolated from broiler flocks in Northern Italy. *Avian. Pathol.* **41**, 579-588.

Griekspoor P., Engvall E.O., Olsen B., Waldenstrom J. (2010). Multilocus sequence typing of *Campylobacter jejuni* from broilers. *Vet. Microbiol.* **140**, 180-185.

Havelaar A.H., Ivarsson S., Löfdahl M., Nauta M.J. (2013). Estimating the true incidence of campylobacteriosis and salmonellosis in the European Union, 2009. *Epidemiol. Infect.* **141**, 293-302.

Humphrey T, O'Brien S, Madsen M. (2007). Campylobacters as zoonotic pathogens: a food production perspective. *Int. J. Food Microbiol.* **117**, 237-257.

Kittl S., Heckel G., Korczak B.M., Kuhnert P. (2013). Source attribution of human *Campylobacter* isolates by MLST and *fla*-typing and association of genotypes with quinolone resistance. *PLoS One* **8**, e81796.

Manning G., Dowson C.G., Bagnall M.C., Ahmed I.H., West M., Newell D.G. (2003). Multilocus sequence typing for comparison of veterinary and human isolates of *Campylobacter jejuni*. *Appl. Environ. Microbiol.* **69**, 6370-6379.

Mughini Gras L., Smid J.H., Wagenaar J.A., de Boer A.G., Havelaar A.H., Friesema I.H., French N.P., Busani L., van Pelt W. (2012). Risk factors for campylobacteriosis of chicken, ruminant, and environmental origin: a combined case-control and source attribution analysis. *PLoS One* **7** (8), e42599.

Ragimbeau C., Schneider F., Losch S., Even J., Mossong J. (2009). Multilocus sequence typing, pulsed-field gel electrophoresis, and *fla* short variable region typing of clonal complexes of *Campylobacter jejuni* strains of human, bovine, and poultry origins in Luxembourg. *Appl. Environ. Microbiol.* **74**, 7715-7722.

Sheppard S.K., Dallas J.F., Strachan N.J., MacRae M., McCarthy N.D., Wilson D.J., Gormley F.J., Falush D., Ogden I.D., Maiden M.C., Forbes K.J. (2009a). *Campylobacter* genotyping to determine the source of human infection. *Clin. Infect. Dis.* **48**, 1072-1078.

Sheppard S.K., Dallas J.F., MacRae M., McCarthy N.D., Sproston E.L., Gormley F.J., Strachan N.J., Ogden I.D., Maiden M.C. (2009b). Campylobacter genotypes from food animals, environmental sources and clinical disease in Scotland 2005/6. *Int. J. Food. Microbiol.* **134**, 96-103.

Wilson D.J., Gabriel E., Leatherbarrow A. J., Cheesbrough J., Gee S., Bolton E., Fox A., Fearnhead P., Hart C.A., Diggle P.J. (2008). Tracing the source of campylobacteriosis. *PLoS Genet.* **4**, e1000203.