Typing of Campylobacter jejuni Isolated from Turkey by Genotypic Methods, Antimicrobial Susceptibility, and Virulence Gene Patterns: A Retrospective Study.

Typing of *Campylobacter jejuni* isolated from turkey by genotypic methods, antimicrobial susceptibility and virulence gene patterns: a retrospective study

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ABSTRACT

In this retrospective study, typing ability, discriminatory power and concordance between typing results obtained on 123 *Campylobacter jejuni* turkey isolates, collected in 1998, within 14 different farms, applying Multi-Locus Sequence Typing (MLST), Pulsed Field Gel Electrophoresis (PFGE), determination of antibiotic resistance profile and virulence gene pattern, were assessed and compared. Overall, 33 Sequence Types (ST), 28 pulsotypes, 10 resistotypes and 5 pathotypes were identified. MLST and PFGE showed the better discriminatory ability (i.e., Simpson's diversity index (DI) > 0.90) as well as unidirectional (i.e., Wallace and adjusted Wallace coefficients > 0.86) and bidirectional (i.e., adjusted Rand coefficient > 0.60) concordance. Moreover, both methods showed a good unidirectional and bidirectional concordance with the resistotype. On the contrary, the congruence of both genotyping methods and resistotype with the pathotype seemed due to chance alone. A clonal relationship was identified among 66.7% of the isolates. 59.7 % of the investigated isolates were resistant to two or more antimicrobials and 91.93% to tetracycline. All the isolates harbored *cadF* and *pldA* genes, whereas a flaA gene product and a cdtB gene product were amplified from 85.4% and 79.7% of the isolates using the primers designed by Bang et al. (2003). 85.4 and 79.7% showed the presence of *flaA* and *cdtB* genes, respectively. The results of this study clarify the level of genetic diversity among the *C. jejuni* originating from turkeys. MLST level of correlation with PFGE, resistotype and pathotype is assessed. This result supports the selection of type and number of typing methods to use in epidemiological studies. Finally, the identification of Clonal Complexes (CC) (i.e., groups of profiles differing by no more than one gene from at least one other profile of the group using the entire *Campylobacter* MLST database) shared between turkey and human isolates suggests that turkeys could be a possible source of *Campylobacter* infection.

INTRODUCTION

In 2013, the prevalence of *Campylobacter* within the European Union was 18.43% in the turkey slaughterhouses, 12.81% in the cutting plants and 10.4 % in turkey meat at retail (EFSA, 2015).

Two features of *Campylobacter* infection have hindered investigations into the epidemiology of human campylobacteriosis: (i) human disease is most commonly sporadic and (ii) the bacterium is widespread and can be readily isolated from the intestine of different animals as well as environmental sources, water and soil (Brown *et al*., 2004). Accurate isolate typing is consequently essential to trace back the source of infection. Among the genotyping methods available for *Campylobacter* typing, Multi-Locus Sequence Typing (MLST) exploits the relative conservation in sequence of certain genes in which variations are more likely to be selectively neutral because of their housekeeping functions. This approach is recognized as the gold standard typing method for this bacterial genus (Sheppard *et al*., 2009). In the past Pulsed Field Gel Electrophoresis (PFGE) was considered the reference method for *Campylobacter* for its high discriminatory power (Maslow *et al*., 1993). The drawbacks of PFGE are the time and labor necessary to perform the protocol properly, and the fact that restriction-based methods are less reproducible and difficult to interpret than sequence-based methods.

Beside molecular methods, different isolates can be characterized according to their antibiotic resistance profile and pattern in virulence genes. The pathogenesis of *Campylobacter* infection is not fully elucidated, although flagella mediated-motility, adhesion to intestinal mucosa, invasion and production of cytotoxin have been identified as virulence determinants (Konkel *et al*., 1997; Wassenaar, 1997, Datta *et al*., 2003). Therefore, four genes, each one responsible for one virulence factor, were investigated in this study; *flaA*, responsible for motility; *cadF*, for adhesion; *pldA*, for invasion; *cdtB*, for toxin production.

When different typing methods are applied to a set of isolates, they should be compared to verify possible correlations in terms of results obtained in order to avoid duplications and to highlight the inability of some of them to provide epidemiological information. Carrico *et al*. (Carrico *et al*., 2006) proposed the use of the adjusted Rand coefficient (AR) as measure for the quantitative assessment of bidirectional correspondence between typing methods taking into account that agreement may arise by chance alone. Severiano *et al*. (Severiano *et al*., 2011) proposed the adjusted Wallace (AW) and corresponding confidence intervals (CI) as quantitative measures of unidirectional congruence between typing methods.

In this research typing ability, discriminatory power and concordance between typing results collected on *C. jejuni* turkey isolates applying MLST, PFGE, determination of antibiotic resistance profile and virulence gene patterns were assessed and compared because (1) the majority of the available knowledge on typing results concerns broiler isolates; (2) more than one isolate was typed from each farm and animal; (3) few publications are available on quantitative assessment of the concordance between typing results applied to the same set of isolates.

MATERIALS AND METHODS

Sampling scheme. In March 1998 a total of 196 turkey caeca were collected in two slaughterhouses that gathered turkeys coming from 14 different farms located in Emilia-Romagna and Veneto regions (northern Italy). At the slaughterhouse, from each farm, 14 animals, belonging to the same group, were randomly selected from the line. Within 5 h after sampling, approximately 5 g of individual caecal contents were diluted 1:1 into saline solution (0.154 M NaCl), homogenized by vortex mixer and diluted further 1:10 and 1:100 in the same solution. Ten microliters of these last two dilutions were then streaked onto two plates of *Campylobacter* Blood-Free Selective Agar (CCDA, Oxoid). The plates were incubated at 37±1°C under microaerobic atmosphere with hydrogen (Bolton *et al*., 1992) and after 48 h of incubation were checked daily up to 5 days for colonies. From each plate, 4 colonies of Gram negative spiral shaped bacteria that looked like *Campylobacter* spp. were sub-cultivated, cloned, submitted to a *Campylobacter* genus specific PCR (Linton *et al*, 1996) and stored at -80 ± 3°C for further typing. In 2014, with the beginning of this retrospective study, the isolates were thawed and submitted to a *C.jejuni-C. coli* specific multiplex PCR (Denis *et al*., 1999). Among the *C. jejuni* isolates, at least one isolate for each animal was selected or more than one based on the colony morphological appearance. Overall, 123 *C. jejuni* isolates were submitted for further investigations.

Multilocus Sequence Typing (MLST). The DNA of the *C. jejuni* isolates was purified using the Chelex® 100 procedure (Biorad, Milan, Italy) and characterized by MLST as previously described (Dingle *et al*., 2001). Allele numbers, Sequence Types (STs), and Clonal Complexes (CCs) were assigned using the PubMLST database (http://pubmlst.org/campylobacter/) (Jolley and Maiden, 2010). The Clonal Complexes (CC) have been defined as groups of profiles differing by no more than one gene from at least one other profile of

the group (Feil, 2004) using the entire *Campylobacter* MLST database. Novel alleles and STs were coded through submission to the MLST database.

Pulsed Field Gel Electrophoresis (PFGE). The *C. jejuni* isolates were typed using PFGE with the use of *Sma*I (Zhou *et al*., 2011). Band patterns were analyzed with BioNumerics 7.5 software (BioNumerics, Applied Maths, Keistraat, Belgium). DNA patterns were compared using the Dice coefficient and the unweighted pair group method with arithmetic averages (UPGMA), with a 1.0% tolerance limit and 1.0 % optimization. Isolates showing a PFGE patterns similarity level of 90% were grouped in the same "PFGE cluster". The PFGE patterns were classified as pulsotypes.

Antimicrobial susceptibility testing. The resistotype (R-type was obtained using the Etest method (AB bioMerieux, Solna, Sweden). Mueller-Hinton Agar II (Becton Dickinson, Franklin Lakes, NJ) containing 5% defibrinated sheep blood was used as medium. The antimicrobial agents tested were ciprofloxacin (CIP), enrofloxacin (EN), gentamicin (G), tetracycline (T), chloramphenicol (C), nalidixic acid (NA), ampicillin (AM) and erythromycin (ER). The plates were incubated at 37 ± 1°C under microaerobic atmosphere for 48 h. *Campylobacter jejuni* ATCC 33560, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were used as control strains. NARMS (2012) cutoff values were used for all the antibiotics tested, except for enrofloxacin and ampicillin, for which the CLSI 2008 cutoff values for *Enterobacteriaceae* and *Campylobacter* were used, respectively. The following resistance breakpoint (µg/ml) were considered: ciprofloxacin ≥ 1, enrofloxacin ≥ 4, gentamicin ≥ 4, tetracycline ≥ 2, chloramphenicol ≥ 32, nalidixic acid ≥ 32, ampicillin ≥ 32 and erythromycin ≥ 8 . The identified R-types were labelled using different numbers.

Detection of virulence genes. The DNA of the *C. jejuni* isolates was amplified by PCR to detect the presence of toxin and virulence genes (*cdtB, cadF, flaA and pldA*), using primers VAT2–WMI-R (5'-GTTAAAATCCCCTGCTATCAACCA-3'; 5'GTTGGCACTTGGAATTTGCAAGGC3'), F2B-R1B (5'-TGGAGGGTAATTTAGATATG-3'; 5'-CTAATACCTAAAGTTGAAAC-3'), *fla*A-F-*fla*A-R (5'-GGATTTCGTATTAACACAAATGGTGC3'; 5'-CTGTAGTAATCTTAAAACATTTTG-3') (Bang *et al*., 2003), pldA-84-Pld-981 (5'-AAGCTTATGCGTTTTT-3'; 5' TATAAGGCTTTCTCCA3') (Datta *et al*., 2003) respectively. *C. jejuni* ATCC 33560 was used as positive control and sterile water as negative control. Each pathotype was labelled as P-type.

Calculation of discriminatory power and congruence among typing methods. The discriminatory indexes (DI) of MLST, PFGE, R-type and pathotype were calculated using the Simpson's diversity index described by Hunter and Gaston (Hunter and Gaston, 1988). The Wallace's coefficient (W), the adjusted Wallace's coefficient (AW) and the adjusted Rand (AR) coefficient, along with the respective confidence intervals (CIs), were all calculated using the Comparing Partitions website (http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Toll). As explained above, W and AW indexes assess the unidirectional concordance between typing methods and AR the bidirectional concordance.

RESULTS

MLST. All the 123 isolates were successfully typed by MLST. Five new alleles were discovered in this study. Overall, the isolates were classified in 33 STs: 11 were unique STs, identified in single isolates, whereas 22 were shared between 2 to 15 isolates. The majority of the common STs (i.e., 59.1%) were shared between isolates collected from turkeys reared in the same farm. The remaining STs were shared between isolates collected from animals housed in different farms, located in the same (i.e., 31.8%) or different regions (i.e., 9.1%). In 71.4% of the tested farms the isolates were characterized by two to four different STs. The most spread ST was ST879, identified among isolates collected in three different farms, followed by STs 50, 227, 3334, 1880, 3476 and 7496 associated to isolates from two different farms. All the remaining STs were identified among isolates coming from a single farm.

PFGE. All the 123 isolates were typed by PFGE. Overall, 28 pulsotypes were associated to the isolates genotyped; 13 were unique pulsotypes, identified in single isolates, whereas 15 were shared between 2 to 26 isolates. The majority of the common pulsotypes (i.e., 53.3%) were shared between isolates collected from turkeys reared in the same farm. The remaining pulsotypes were shared between isolates collected from animals housed in different farms, located in the same (i.e., 20%) or different regions (i.e., 26.7%). In 57.1% of the tested farms the isolates were characterized by two to four different pulsotypes. The most spread pulsotypes were those labelled as 1 and 30, identified among isolates collected in five different farms, followed by pulsotype 11, associated to isolates from three farms, and pulsotypes 3, 14, 15 and 28, associated to isolates from two different farms. All the remaining pulsotypes were identified among isolates coming from a single farm.

Resistotype (R-type). The distribution of the Minimum Inhibitory Concentration (MIC) values of the 123 isolates tested is shown in Table 1. All the 123 isolates of *C.jejuni* were sensitive to gentamicin and chloramphenicol whereas four to all antimicrobials tested. Furthermore, 92% of the tested isolates were resistant to tetracycline, 55% to nalidixic acid, 52% to enrofloxacin and ciprofloxacin, 13% to ampicillin. Finally, one isolate was resistant to erythromycin. Overall, according to the resistance to antimicrobials, 10 different R-types were identified and labelled with numbers (Figure 1). The R-type 1 (T) was found in 35% of the isolates; R-type 2 (CIP-EN-T-NA) in 43%; R-type 3 (CIP-EN-NA) in 2%; R-type 4 (AM) in 2%; R-type 5 (T-NA) in 3%; R-type 6 (T-AM) in 4%; R-type 8 (CIP-EN-T-NA-AM) in 6%; R-types 7 (CIP-EN-T-NA-ER) and 9 (CIP-EN-NA-AM) in 1% of the isolates each; R-type 11 (S), referred to *C.jejuni* susceptible to all antibiotic tested, in 3% of the isolates.

Pathotype (P-type). All the 123 *C. jejuni* isolates harbored the *cadF* gene responsible for adherence. The presence of *pldA* gene, responsible for invasion, was highlighted in all isolates, while the *flaA* gene, involved in isolate motility, was determined in 105 isolates (85.4%). As regards *cdtB* gene, associated with toxin production, it was present in 98 (79.7%) *C. jejuni* isolates. Totally, 5 different P-types, labelled as numbers, were identified according to the pathotype (Figure 2). The P-type 10 (*cadF- flaA- pldA - cdtB*) was identified in 68% of the isolates, followed by P-types 4 (*cadF – flaA- pldA*) (16%) and 5 (*cdtB - cadF - pldA*) (11%). The two remaining P-types, 7 (*cadF-pldA*) and 3 (*cadF - flaA*) were identified in 4 and 2 isolates, respectively.

Isolate discrimination. The discriminatory indexes (DI) of the applied typing methods ranged between 0.946 for MLST and 0.499 for P-type (Table 2). Both MLST and PFGE showed a DI higher than 0.90. Therefore, they both show a probability to discriminate *C. jejuni*

isolates different from a genetic point of view higher than 90%. The DIs calculated for each different typing method were significantly different ($p<0.001$).

Concordance among typing methods. The Wallace's coefficient (W) and the adjusted Wallace coefficient (AW) concerning unidirectional concordance between MLST, PFGE, R-type and P-type (Table 3) showed a strong concordance (i.e., W and AW > 0.86) between MLST and PFGE, as well as a good concordance (i.e., W and AW > 0.73) between MLST and R-type. As explained in the introduction, this result means that the classification, in terms of different or identical type strains obtained using MLST can predict that found using PFGE and R-type. A sufficient level of concordance (i.e., W and AW > 0.43) was observed also between PFGE and both MLST and R-type (Table 3). Finally, a very low degree of concordance (i.e., W and AW > 0.097) was found between R-type and both MLST and PFGE. The P-type did not show concordance with the other typing methods, meaning that agreements between the results provided by P-type and one of the other methods is due to chance only (Table 3). In fact in Table 3 the W_i values calculated for the unidirectional concordance between P-type and MLST, PFGE and R-type are always included in the CI of the W coefficient for each combination of typing methods. Therefore, all the AW values are very low (Table 2). The results on the bi-directional concordance showed an AR > 0.60 between MLST and PFGE only (Table 4). This AR value was significantly higher ($p<0.001$) than the AR values for PFGE and R-type (AR 0.164) and MLST and R-type (AR 0.171). Finally all the AR values between P-type and all the other typing methods were significantly lower ($p<0.001$) of the AR values between the other combinations of typing methods.

Type strains identified. The isolates tested were assigned to different type strains according to their ST, R-type, pulso-type and P-type (Table 5). Clonal relationships, referred to isolates belonging to the same type strain, were identified for 66.7% of the tested farms (Table 5). They referred mainly to isolates from the same farm. However, isolates belonging to the same type strain isolated in different farms of the same region were also detected (Table 5). Overall, the number of type strains identified within the same farm ranged between 2 and 8.

DISCUSSION

In this study MLST and PFGE showed the better discriminatory ability, followed by R-type and P-type. Moreover, MLST and PFGE showed a good unidirectional and bidirectional concordance. A good unidirectional concordance was also observed between MLST and R-type, PFGE and both MLST and R-type, as well as R-type and the two investigated genotyping methods. These results seems to suggest that MLST only can be applied to trace clonal relationships between isolates. However, the isolates classified in the ST 3476, obtained in farms 5 and 15 displayed different pulso-types and R-types; therefore they would be improperly classified as clonal by MLST only. The same consideration can be extended to isolates with ST 50, collected in the farms 7 and 19, as well as to the isolates with ST 7496, from farms 6 and 52. This picture is important in order to approach the analysis of different typing data when available. However, the most promising technique in the analysis of outbreak-associated isolates, leading to faster and more precise source identification and to discriminate between alternative epidemiological hypotheses is currently represented by whole-genome sequencing (WGS) (Revez *et al*., 2014; Harrison *et al*., 2013).

206 The CC21 identified in 23.38% of the turkey isolates included in this study has been previously described for chicken *C. jejuni* isolates
207 (Ragimbeau *et al*., 2009; Sheppard *et al*., 2009; Colles and Maiden, 2012; Kittl *et al*., 2013). Moreover, additional CCs, like CC443 and
208 CC446, associated to 7.25 and 1.61% of the turkey isolates were already identified among chicken strains (Ragimbeau *et al*., 2008; Kittl
209 *et al*., 2013). Interestingly, both CC21 and CC443, along with CC206 and CC257, associated to 4.84 and 12.09% of the turkey isolates,
210 respectively, were already identified among human *C. jeuni* isolates and have been frequently associated with human diseases
211 (Ragimbeau *et al*., 2008; Sheppard *et al*., 2009; de Haan *et al*., 2010; Colles and Maiden, 2012). The identification of CC shared between
212 turkey and human isolates suggests that turkeys could be a possible source of *Campylobacter* infection.
213
214 The 59.7 % of the investigated isolates were resistant to two or more antimicrobials. Although the turkey isolates investigated in this
215 study were collected before the adoption of EU Regulation 1831/2003 banning the use of antibiotics as growth promoters in animal feeds,
216 high occurrences of multidrug-resistant in *C. jejuni* isolated from turkeys have been reported more recently. A high prevalence of
217 tetracycline resistance in *C. jejuni* isolates from chickens and turkeys has been described (Luangtongkum *et al*., 2009; Andersen *et al*.,
218 2006; Gu *et al*., 2009; Zhao *et al*., 2011). In this study high resistance rates to (fluoro)quinolones were also observed. High level of
219 tetracycline and (fluoro)quinolones resistances were also reported in *C. jejuni* human, animals and food isolates in 2013 (EFSA, 2015).
220 In regard to the detection of virulence genes, the pathotype results do not correlate to other methods employed in this study; however,
221 this study contributes to the limited data available on virulence genes of *Campylobacter jejuni* isolates from turkeys (Bang *et al*., 2004;
222 Thorsness *et al*., 2008). The occurrence of the virulence genes observed in this study is in agreement with previous surveys performed on
223 *C. jejuni* isolated from turkeys where *cadF*, *pldA* and *flaA* genes resulted the most conserved genes.The majority of our isolates were

positive for all four virulence genes in agreement with that observed by Thorsness *et al*. (2008), suggesting a potential ability of these *Campylobacter* to colonize turkeys and a potential capability to induce human *Campylobacter* infection (Konkel *et al*., 2000). However infection is a multifactorial process and studies on the regulation and expression of these genes *in vitro* and *in vivo* will be needed to better understand the role of these genes in the pathogenesis of campylobacteriosis.

CONCLUSIONS

The results of this study show that the population of *C. jejuni* isolates colonizing turkeys includes different type strains within the same farm, which must be discriminated in order to make source attribution in case of human campylobacteriosis link to turkey or turkey products. Therefore, a representative number of animals and isolates must be collected during epidemiological investigations. Even if we already entered the WGS era, whenever an alternative typing method must be selected for typing *C. jejuni*, MLST is confirmed as the best choice. In fact, in this study MLST shows the best discriminatory ability and good correlation with PFGE and R-type. Even if this result seems to indicate to avoid to run all methods on the same isolates, isolates sharing the same MLST profile with different PFGE and R-type were identified.

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