Environmental Research 127 (2013) 56–62

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Multilocus sequence typing of *Campylobacter jejuni* and *Campylobacter coli* strains isolated from environmental waters in the Mediterranean area

S. Rodríguez-Martínez, S. Cervero-Aragó, I. Gil-Martin, R. Araujo *

Departament de Microbiologia, Universitat de Barcelona, Av. Diagonal 643, Barcelona 08028, Spain

ARTICLE INFO

Article history:
Received 18 April 2013
Received in revised form
22 August 2013
Accepted 2 October 2013
Available online 8 November 2013

Keywords:
Campylobacter jejuni
Campylobacter coli
MLST
River
Urban sewage and animal sewage

ABSTRACT

Campylobacter jejuni and *Campylobacter coli* are important animal-related waterborne pathogens that are distributed worldwide. To further understand *Campylobacter* populations in water from the Mediterranean area, the genetic diversity of environmental strains was analyzed using multilocus sequence typing (MLST). MLST was also used to determine the potential geographical differences between these bacterial strains and other campylobacters isolated worldwide. The typing study was conducted using 58 strains isolated from the Llobregat river and other water sources, such as urban sewage, animal wastewater and clinical samples. Thirty-nine different sequence types were obtained; eight of these sequences were described for the first time in this study, suggesting the presence of local strains. The identified *C. jejuni* strains were the most diverse population, whereas the identified *C. coli* strains showed a high clonal structure, which clustered most of the sequence types into a few clonal complexes. The strains were not exclusively related to specific water sources. However, comparing the identified strains with an international database showed that most of the Mediterranean strains that were exclusively isolated from environmental waters have previously been isolated from similar sources, particularly those obtained from river water. Additional studies, including those in different geographical areas using a wide range of *Campylobacter* sources, are required to improve the global knowledge concerning *Campylobacter* dissemination in the environment.

1. Introduction

Thermotolerant *Campylobacter* species are the major cause of bacterial gastroenteritis in Europe and most developed countries worldwide (Ailes et al., 2008; European Food Safety Authoriry, 2010; Mickan et al., 2007). It is estimated that over 95% of *Campylobacter* spp. infections are caused by *Campylobacter jejuni* and *Campylobacter coli* (Butzler, 2004; Nachamkin et al., 2000). These pathogens are typically located in the intestinal biota of many warm-blooded animal species, particularly birds (Newell et al., 2001; Skirrow, 1994), which excrete these bacteria in feces and pollute nearby environments. Food produced from these animals and polluted waters are considered major sources of *Campylobacter* transmission to humans (Koenraad et al., 1997; Savill et al., 2003).

Although *Campylobacter* are not considered aquatic microorganisms, many species have been isolated from river water worldwide, and the presence of these bacteria is considered a sign of recent fecal contamination (Jones, 2001). Common sources of pollution include the discharge of effluents from untreated urban sewage, local food industries, agricultural run-off and livestock waste, and other environmental inputs, such as those from wild animals or birds living close to the water source (Hokajarvi et al., 2013).

Most studies on the presence and distribution of *Campylobacter* species have been conducted in Northern European countries, including the UK, USA and New Zealand (Devane et al., 2005; Eyles et al., 2003; Obiri-Danso and Jones, 1999; Sails et al., 2002; Savill et al., 2001; Vereen et al., 2007). Information regarding the occurrence of *Campylobacter* in the Mediterranean area is scarce. Indeed, our group has previously published one of the few studies focused on this area (Rodríguez and Araujo, 2010). In a 2-year study, we monitored the occurrence of *Campylobacter* species in the Llobregat River and at the most probable sources of pollution in the surrounding area, including poultry wastewater, pig slurries and urban sewage (Hundesa et al., 2009). In this work, strains from those origins, together with some clinical strains, were analyzed in order to see whether a genetic relationship existed between these microorganisms.

Multiple methods can be used to determine epidemiological relationships between microbial isolates (Foley et al., 2009). Different typing methods have been described for *Campylobacter* (Wassenaar and Newell, 2000). The most frequently used are

* Corresponding author. Fax: +34 934039047.
E-mail address: raraujo@ub.edu (R. Araujo).

0013-9351/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.envres.2013.10.003

pulse-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). MLST is a technique based on sequencing gene fragments of housekeeping loci. This discriminatory procedure has some advantages over other molecular methods, such as the unambiguous nature of the nucleotide sequence data, the employment of reproducible technology (Maiden et al., 1998) and the fact that data can easily be shared electronically between laboratories, facilitating comparisons of *Campylobacter* sequences from strains isolated worldwide (http://pubmlst.org/campylobacter/) (Dingle et al., 2005; Foley et al., 2009).

Thus, using MLST, we addressed the following aims of the investigation: (i) to type and analyze the genetic diversity of the *Campylobacter* strains collected from environmental waters and some clinical samples; (ii) to compare the Mediterranean strains with those from the international database; and (iii) to add our collection of strains isolated from the Mediterranean area to the existing database.

2. Material and methods

2.1. Campylobacter isolates

Campylobacter strains were isolated during a 2-year study of environmental water in a Mediterranean area (Rodríguez and Araujo, 2010). Briefly, appropriate volumes of the water samples were analyzed using the most probable number (MPN). The samples were concentrated and inoculated in tubes containing the Preston enrichment broth (Oxoid, Basingstoke, UK) and incubated at 42 °C for 48 h under microaerobic conditions. The samples were subcultured from the Preston broth tubes to the Karmali agar plates (Scharlab, Barcelona, Spain) and incubated for 48 h at 42 °C under microaerobic conditions using the Oxoid CampyGen™ system (Oxoid, Basingstoke, UK). The plates were examined for the presence of *Campylobacter* colonies, and the isolates were identified as *C. jejuni* or *C. coli* using multiplex PCR according to the methods of Wong et al. (2004). The strains were stored at −80 °C in Brain Heart Infusion broth (Difco, New Jersey, USA) containing 20% glycerol.

A total of 26 *C. jejuni* and 32 *C. coli* isolates were typed. The samples comprised 21 isolates from Llobregat river water (6 *C. jejuni* and 15 *C. coli*), 18 isolates from urban sewage water (7 *C. jejuni* and 11 *C. coli*), 8 *C. jejuni* strains from poultry wastewater and 5 *C. coli* strains from pig slurry. Six strains (5 *C. jejuni* and 1 *C. coli*) from clinical cases of gastroenteritis were also included in the analysis as an outside group not isolated from water. The samples were in-kind donations from Dr. Bartolomé from the Vall d´Hebron Hospital of Barcelona. All strains were cultured on Karmali agar plates (Scharlab, Barcelona, Spain) at 42 °C for 2 days under microaerobic conditions in a jar using the Oxoid CampyGen™ system (Oxoid, Basingstoke, UK). Genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, Wis.).

2.2. Multilocus sequence typing

Multilocus sequence typing (MLST) of seven housekeeping genes (aspA, glnA, gltA, glyA, pgm, tkt and uncA) was performed according to previously described protocols (Devane et al., 2005; Dingle et al., 2001). The DNA was amplified using the Illustra™ PureTaq Ready-to-Go PCR Bead Kit (GE Healthcare, Buckinghamshire, UK). The PCR amplicons were purified using the Invisorb Fragment Clean Up kit (Invitek GmbH, Germany). The nucleotide sequences were determined using the BigDye Terminator v2 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA). The reaction products were detected using an ABI Prism 3700 Capillary Sequencer (Applied Biosystems, USA).

The sequence files were edited using BioEdit v. 7.1.3 software (Hall, 1999). The allele numbers, sequence types (STs) and clonal complexes were assigned through comparison of the obtained sequences with the *Campylobacter* MLST database (http://pubmlst.org/campylobacter/) (Jolley and Maiden, 2010). Novel STs were submitted to this MLST database for number designation. Information concerning *Campylobacter* strains isolated worldwide was also obtained from the same database.

Neighbor-joining trees of the concatenated gene sequences of each isolate were constructed using MEGA v. 5.0 (Tamura et al., 2011) with Kimura 2 parameters and 1000 bootstrap simulations.

3. Results

3.1. Sequence typing of Campylobacter strains

Strains from five different origins (Llobregat river water, urban sewage, poultry wastewater, pig slurries and clinical samples) were analyzed. The results of the MLST analysis are summarized in Table 1. A total of 39 different STs were identified among the 58 strains analyzed. Among these, 20.5% of the STs were identified for the first time and submitted to the database (Table 2). These sequences originated from new combinations of previously described alleles.

A total of 22 different STs were detected among the 26 *C. jejuni* strains. Most of the *C. jejuni* STs occurred only once in the dataset, except for ST-50, which occurred four times (three clinical strains and one urban sewage strain), and ST-441, which occurred twice (one river water strain and one urban sewage strain). *C. coli* presented lower ST diversity, with 17 STs among the 32 isolated strains. The predominant *C. coli* STs were ST-825, which was isolated from river water and pig slurry, and ST-1771, which was isolated from river water. The predominant STs differed depending on the water origin. ST-1771 and ST-1766 were the predominant isolates in river water (5/15 and 4/15 isolates, respectively), ST-825 was the predominant isolate in urban sewage (4/11), and a similar number of STs were isolated from pig slurries (1/5).

A comparison of our data with the *Campylobacter* MLST database resulted in the assignment of STs to a ST-clonal complex (Table 1). ST-clonal complex groups STs that share four or more alleles. Among the 22 *C. jejuni* STs, eight of them could not be assigned to any complex. Only six STs are grouped in the same two clonal complexes: ST-complex 45 and ST-complex 21. Four STs are grouped in ST-complex 45: ST-45 and ST-5168, isolated from urban sewage, and ST-2663 and ST-3730, both isolated from poultry wastewater. And two STs are grouped in ST-complex 21: ST-50, isolated from urban sewage and clinical samples, and ST-760, isolated from poultry wastewater.

Most of the *C. coli* STs obtained (11/17) were assigned to clonal complex ST-828. These STs were ST-1009, ST-1689 and ST-5169, which were isolated exclusively from urban sewage; ST-890, ST-1595 and ST-2713, which were isolated from pig slurries; ST-825 and ST-854, which were isolated from urban sewage and pig slurries; and ST-827, which was isolated from urban sewage and clinical samples. Notably, none of the strains isolated from river water belonged to the 828-complex. The remaining nine STs obtained could not be assigned to any clonal complex.

3.2. Phylogeny and clustering of the isolates

A neighbor-joining tree was constructed using the 3309-bp concatenated MLST allele sequences for the two species of *Campylobacter* analyzed; bootstrapping values of 100% were obtained for the branch separating the two species after 1000 repetitions (data not shown). Therefore, two new neighbor-joining trees were constructed according to species (Fig. 1).

We obtained a tree that divided the *C. coli* isolates into two large groups. The first cluster grouped the isolates obtained from river water and urban sewage. The relationship between these STs was highly variable; remarkably, the ST-4356 obtained from river water was the most genetically divergent sequence. The second major cluster grouped the isolates collected from urban sewage, pig slurries and a clinical sample as well as the reference strain. The bootstrap values within this second cluster were higher than those in the first cluster of STs.

The tree obtained for *C. jejuni* STs showed higher divergence between the STs than the *C. coli* tree. Although there was no clear division into clusters according to ST origin, various patterns were observed. The tree showed three different associations between poultry wastewater and urban sewage STs: ST-3730 with ST-45, ST760 with ST-50, and ST-305 with ST-969. We also observed a genetic relationship between ST-572 and ST-429, which were isolated from river water and poultry wastewater, respectively.