Genetic diversity and virulence potential of *Campylobacter coli* and *Campylobacter lanienae* carried by wild boars from the metropolitana area of Barcelona

MASTER'S DEGREE IN ZOONOSES AND ONE HEALTH

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CReSA IRTA Centre de Recerca en Sanitat Animal

UAB Universitat Autònoma de Barcelona

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ABSTRACT

Campylobacter spp., are the main cause of human gastroenteritis in Europe and in USA, with poultry as the main source of infection. Other sources of infection exist, including wild fauna, but its contribution to human infection is still poorly known. The presence of wild boars is growing in urban environments, and consequently the risk of transmission of zoonotic pathogens is increasing as well. To gain insight into the epidemiology and virulence potential of *Campylobacter spp.*, *C. coli* and *C. lanienae* isolated from wild boars circulating in the Metropolitan area of Barcelona were analyzed. The isolates had previously been typed by PFGE and isolates representative of the different pulsotypes have been analyzed in this study by MLST and screened by PCR for the presence of putative virulence factors. A high genetic diversity was observed amongst both *Campylobacter* species. A total of 12 different STs, of which 4 were novel, were identified among *C. coli* isolates; ST 854, belonging to the clonal complex CC 828 was the most prevalent. All *C. lanienae* isolates (unless four alleles; *uncA* 7, *uncA* 12, *glnA* 12, *glyA* 21) belonged to novel STs and to determine the relationship among them, a phylogenetic tree was constructed using concatenated MLST loci. Three *C. lanienae* isolates from two different areas showed 100% similarity and whilst two other isolates were also closely related and differed only in 6 nucleotides. In both *Campylobacter* species some genotypes (ST 854, ST 827, ST 9235 in *C. coli*) were found in the different studied areas, indicating a possible circulation of those strains among wild boars from urban and natural areas (Collserola massif and UAB campus). Besides the novel STs, all other *C. coli* STs have been associated with human gastroenteritis. Some virulence genes like *flaB*, *ceuE* and *hcp* have been detected in the majority of the isolates whereas the prevalence of other genes (*ciaB*, *racR* and *wlaN*) was low. In addition, some isolates were positive for 12 of the 14 virulence genes analyzed, suggesting a high pathogenic potential.

Our results show that wild boars from the metropolitan area of Barcelona are carriers of *Campylobacter* genotypes associated with human gastroenteritis that also show a high virulence potential. These findings are of public health concern because of the increasing contact of these wild boars with humans in the studied areas.

1. INTRODUCTION

1.1. Zoonoses

Zoonoses are diseases of animal origin that can be transmitted naturally from animals to humans or vice versa, as a consequence of direct contact, indirect environmental contact, or through consumption of contaminated food. Among all human pathogens, almost 60% are zoonotic, and they can be viruses, parasites, bacteria, prions and fungi (Chlebicz and Śliżewska, 2018). Zoonoses represent a threat for global health as they can affect people, domestic animals and wildlife causing serious health emergencies worldwide. Globalization, climate change and changes in human behavior, give these pathogens numerous opportunities to colonize new species and evolve into new forms (Cunningham et al., 2017), increasing the chances of causing an outbreak in human populations.

In the European Union (EU) campylobacteriosis is the most commonly reported zoonosis and with salmonellosis are the main causes of food-borne illnesses (EFSA, 2017). These bacteria frequently colonize the intestinal tract of healthy food-producing animals or wild animals that usually do not develop any clinical sign (Navarro-Gonzalez et al., 2014). However, in humans and in some domestic animals the infection by these bacteria is characterized by severe abdominal pain and diarrhea which can be fatal among very young children, elderly and immunosuppressed individuals (Bosco-Lauth et al., 2018). Moreover, the economic costs associated with foodborne disease can be severe on people, food companies, and country reputation.

The main causes of human infections by foodborne bacteria are unhygienic practices in food production, harvesting and preparation (Adley and Ryan, 2016). Most emerging pathogens have originated on wildlife spillover into livestock and human hosts, as a result of established wildlife populations around human settlements (Cunningham et al., 2017). In addition, *Campylobacter* transmission between wildlife and farm animals may be bidirectional (Navarro-Gonzalez et al., 2014), and thus livestock and wildlife constitute a source of pathogen infections for humans.

Wild boars are an example of wild species that harbor a wide variety of zoonotic pathogens that can be shared with livestock, pets and humans (Bosco-Lauth et al., 2018) (Ruiz-Fons, 2017). Zoonotic enteric pathogens found in wild boars, like *Campylobacter* species are unlikely to result in a human pandemic. However, they are capable of

inducing sporadic human cases (Bosco-Lauth et al., 2018) which is an important public health issue.

1.2. Wild boars

Wild boars are a prolific species throughout the world with and enormous plasticity and ability of adaptation to new situations. As a result, their populations are expanding to new areas and increasing significantly in number (Castillo-Contreras et al., 2018). Their presence poses significant threats to native wildlife populations, ecological habitats, agricultural practices and zoonotic disease transmission (Bosco-Lauth et al., 2018).

Wild boar has an omnivorous and opportunistic diet (Trani, Ford and Chapman, 2007). They eat what it is available which can vary with seasons, weather conditions and locations. They mainly eat crops, fruits, nuts and roots but they can also consume small rodents, insects and worms (Palomo, Gisbert and Blanco, 2007). Because of their omnivorous foraging tendencies, they can promote erosion, soil deterioration and crop destruction which can lead to huge economic losses especially for farmers (Geisser et al., 2004).

Wild boars are cosmopolitan species and have one of the widest geographic distributions of all terrestrial mammals (Albarella et al., 2009). They are commonly found through-out Eurasia and North Africa. In America, New Zeeland and in some Islands of the Pacific they have been introduced by humans (Palomo, Gisbert and Blanco, 2007). As they are wide-spread distributed, they inhabit diverse habitats from semi-desert to tropical rain forest, temperate woodlands, grasslands and reed jungles among others. At global level, there are no major threats to the species and at global population estimate, numbers can be high in many places, so it is not considered an endangered species (Oliver and Leus, 2018).

Wild boars have been described widely thought the Iberian Peninsula. Density varies from 1 individual/km^2 to 15 individuals/km^2, with enormous fluctuations during the year. In recent years, there has been an increase in their populations in the Iberian Peninsula, similarly to what is also happening within the European populations (Palomo, Gisbert and Blanco, 2007). In the case of Barcelona (northeastern Spain), the presence of wild boars was scarce since the 80s, when following the global trends wild boars started to increase their presence in urban areas. This population of wild boars that

enter into the city are mainly from Collserolla, a natural massif that surrounds the north west of Barcelona (Castillo-Contreras et al., 2018) (Navarro-Gonzalez et al., 2013).

The expansion of wild boars throughout the world may be explained by many factors such as their rapid reproductive rate, their highly adaptability specially in human altered landscapes, the loss of predators, hunting pressure and their flexible and opportunistic diet (Bosco-Lauth et al., 2018). Furthermore, it has been suggested that climate change, through decreasing winter severity, could increase wild boars survival, contributing to the growth of the species (Ruiz-Fons, 2017). Consequently the species has naturally colonized new areas specially peri-urban and urban areas like the city of Barcelona intensifying conflicts with humans (e.g. traffic accidents, damage of streets and parks…) and farming activities (e.g. destruction of crops, fences…) (Castillo-Contreras et al., 2018) (Geisser et al., 2004).

Wild boars are a source of zoonotic pathogens for livestock, pets and humans (Bosco-Lauth et al., 2018) (Ruiz-Fons, 2017). In some cases wild boars can act as true reservoirs of pathogens (e.g. hepatitis E virus or Japanese encephalitis virus), whereas in other cases they are only intermediate hosts or transport hosts (e.g. *Salmonella spp.*, *Campylobacter spp*.) (Ruiz-Fons, 2017).

Changes of human habitation to suburban areas, increased use of lands for agricultural purposes, and deforestation have increased chances of contact exposure of wild boars with domestic animals and humans (Meng et al., 2009). Outdoor ranging domestic pigs are especially vulnerable to be infected with diseases carried by wild boars, and consequently farmers or people that consume their meat (raw or improperly cooked) can be infected with these pathogens (Wacheck et al., 2010). Additionally, wild boars defecate in public spaces and drink water from public fountains and swimming pools increasing the probabilities of disease transmission. Finally, wild boar hunting is another perfect opportunity for direct human contact with wild boars, and incorrect management practices of its meat can lead to human infections (Meng et al., 2009) (Wacheck et al., 2010).

1.3. *Campylobacter*

Campylobacter is a genus of microaerophilic, gram negative, spirally curved and small bacilli that do not form spores (Allos, 2001). They are motile by means of unipolar or bipolar flagella and they can be found in a wide variety of habitats due to their high

adaptability to various environmental niches (Allos, 2001) (Lee and Newell, 2006). At present, the genus *Campylobacter* is divided into 39 distinct species and 16 subspecies (*List of prokaryotic names with standing in nomenclature*, 2018). Thermophilic *Campylobacter* spp., are the most frequent identified bacteria causing diarrhea in the developed world (Chlebicz and Śliżewska, 2018) (Navarro-Gonzalez and Ugarte-Ruiz et al., 2014). Nevertheless, other species colonize the gastrointestinal tract of many animal species without being pathogenic (Wilkinson et al., 2018). Every year 1 out of 10 people suffer from campylobacteriosis and 33 million of healthy life years are lost (WHO, 2012). In 2016 campylobacteriosis was the most commonly reported zoonosis in the EU (EFSA, 2017).

Between 2008 and 2016 there had been a significant increasing trend in the number of cases of campylobacteriosis in the EU, reflecting several improvements in the surveillance system and reporting systems, improved diagnostic facilities and public awareness (EFSA, 2017). The typical symptoms of the disease are fever, diarrhea (often bloody) and severe abdominal pain. By its clinical characteristics it is impossible to distinguish from gastrointestinal infections produced by other foodborne bacteria like *Salmonella* (Allos, 2001). To confirm the causative agent, the clinical diagnosis is complemented with stool cultures and polymerase chain reaction (PCR) or biochemical tests to identify the isolate at species and subspecies level (EFSA, 2017).

In most cases campylobacteriosis is a self-limiting disease but up to 10% of cases may require medical intervention (Lee and Newell, 2006). Serious systemic illness caused by *Campylobacter* infection rarely occurs, yet in some patients, especially those at high risk (children, elderly or immunocompromised people) it can lead to sepsis and death (Allos, 2001). *Campylobacter* infection is also associated with an infrequent but serious sequelae, such as Guillain-Barré syndrome (GBS, an acute demyelinating disease of the peripheral nervous system) or the Miller-Fisher syndrome (Endtz et al., 2000). Although the risk of developing GBS after an infection with *Campylobacter* is actually quite low, it has been seen that GBS is more severe when it is preceded by *Campylobacter* infection rather than if it is not (Allos, 2001) (Nachamkin et al., 1998).

The most important route of *Campylobacter* infection is the consumption or improper handling of chicken. There are other sources of infection like unpasteurized milk, improperly cooked meat, raw meat and water. As heat kills the bacteria, cooking and basic food hygiene practices are the main important measures for preventing the disease (Allos, 2001).

The majority of campylobacteriosis are caused by *Campylobacter jejuni*. Only 10% of cases are caused by *Campylobacter coli* and less than 1% are due to *Campylobacter lari*. Gastrointestinal disease caused by other *Campylobacter* species (like *C. lanienae* and *C. upsaliensis*) are less frequent (Allos, 2001) (Wilkinson et al., 2018).

In 2000 *C. lanienae* was isolated for the first time in fecal samples of asymptomatic abattoir workers (Logan et al., 2000) and the first case of a symptomatic human infection with *C. lanienae* was reported in 2016 (Lévesque et al., 2016). As *C. jejuni* and *C. coli* are the most isolated species, they are the most studied ones, and surveillance studies, epidemiologic investigations and laboratory research are focused primarily on them. On the contrary, the epidemiology and the clinical role of *C. lanienae* or other less common species in human and animal diseases is much less understood (Cáceres et al., 2017). Furthermore, the real prevalence of this uncommon emerging species may be underestimated due to their fastidious growth characteristics along with the methods used for culture, which usually favor the growth of other species (*C. jejuni* and *C. coli*). Nevertheless, the number of emerging *Campylobacter* species isolated in human and animal samples is growing every day (Navarro-Gonzalez and Ugarte-Ruiz et al., 2014), which shows the importance that these species are acquiring nowadays as potential cause of human diarrheal disease, pointing the need of further investigations in this line.

Birds are considered to be the natural host of thermophilic *Campylobacter* spp. because their body temperature is the optimum for their growth (Lee and Newell, 2006). However, as stated above, *Campylobacter* spp. are widely distributed among all warm-blooded animals and they have also been isolated from shellfish (WHO, 2012). In particular, *C. jejuni* is more associated with chickens, cattle and sheep whereas *C. coli* is associated with pigs, sheep and poultry. *C. lanienae*, a member of the "emerging *Campylobacter* spp.", has been isolated mainly from swine, but also from wild boars, wild ruminants, sheep and bovine (Lévesque et al., 2016) (Guevremont et al., 2008) (Schweitzer et al., 2011).

Although wild boars have been described as carriers mainly of *C. coli* and *C. jejuni*, the prevalence of other emerging species like *C. lanienae* is growing. Moreover, *Campylobacter spp*. have been isolated from wild boar meat so the potential of entering to the food chain through this route is high (Navarro-Gonzalez et al., 2014). In many studies the bacteria isolated from wild boars was a species that had been associated with human disease suggesting that wild boars are of concern for transmitting these enteric pathogens to humans (Bosco-Lauth et al.,2018).

1.4. Molecular epidemiology

Over recent years, a wide variety of typing and subtyping techniques have been developed in order to characterize bacterial isolates and classify them at the species or subspecies level. The main purposes of bacterial subtyping are to evaluate taxonomy, define phylogenetic relationships, examine evolutionary mechanisms and to conduct epidemiological investigations (Struelens et al., 2001). In the case of *Campylobacter*, the typing of bacterial isolates is useful for tracing the source and routes of infection, which is key to design effective measures to control and prevent further infections (Dingle et al., 2002).

Initially, characterization of bacterial isolates was based on phenotypic characteristics such as morphology, biochemical and serological properties. However, these methods are generally too variable and the results are often ambiguous and in most cases, do not resolve the epidemiology of the strain (Ranjbar et al., 2014). At present, the most used methods for typing *Campylobacter* or any other bacteria are genotyping techniques, which are based on DNA methods such as polymerase chain reaction (PCR) methods, restriction fragment length polymorphism (RFLP) or pulse-field gel electrophoresis (PFGE) (Ranjbar et al., 2014) (Wassenaar and Newell, 2000), among others. Among these, PFGE is a technique extensively used to characterize genetically *Campylobacter* spp.

In PFGE, bacterial chromosomes are digested using restriction enzymes obtaining various large DNA fragments. The resultant digested DNA is separated depending on size using special electrophoretic conditions. For each isolate a different banding pattern is obtained. These patterns are called macrorestriction profiles or pulsotypes which are the reflection of variations in restriction sites (Wassenaar and Newell, 2000). Differences between pulsotypes are compared among isolates which are used for epidemiological investigations (Ranjbar et al., 2014). Nevertheless, the results obtained with the previous methods are not easy to compare within different laboratories so to solve this problem, a new technique, multilocus sequence typing (MLST) was developed (Maiden et al., 1998).

MLST uses comparative DNA sequencing of conserved housekeeping genes to characterize organisms (Maiden et al., 1998). This technique is a highly reproducible method that allows simple worldwide comparison of the obtained results as it uses an universal nomenclature for the nucleotide sequence data (Korczak et al., 2009). Dingle et al., developed the first *Campylobacter* MLST system to characterize *C. jejuni* strains and investigate *C. jejuni* cases (Dingle et al., 2001). Since then and thanks to the

availability of draft genomes of more *Campylobacter* spp., MLST methods for other *Campylobacter* spp. were developed. In 2005, the method was extended by Miller et al., to type *C. coli*, *C. lari*, *C. upsaliensis* and *C. helveticus* (Miller et al., 2005) and some years later a MLST method for the emerging *Campylobacter* spp. (*C. hyointestinalis*, *C. lanienae*, *C. sputorum*, *C. concisus* and *C. curvus*) was also constructed (Miller et al., 2012).

The MLST method developed for *Campylobacter* spp. amplifies and sequences 7 conserved housekeeping genes (Table 1). Sequences of all 7 locus are obtained, and for each unique allele sequence, a different allele number is given. The combinations of these 7 allele numbers constitute an allelic profile or sequence type (ST) which is also identified with a number. Those isolates that share at least 4 alleles in common are grouped under a common central genotype known as the clonal complex (CC) genotype (Miller et al., 2005).

Table 1. Genes used in MLST for *C. coli* and *C. lanienae* (Miller *et al.*, 2005) (PubMLST *Campylobacter*).

Locus	Name	Function	Fragment size
aspA	Aspartase A	Aminoacid metabolism	477
glnA	Glutamine synthetase	Aminoacid metabolism	477
gltA	Citrate synthase	Tri carboxylic acid cycle	402
glyA	Serine hydroxy methil transferase	Energy metabolism	507
pgm/glmM	Phospo glucosamine mutase	Aminoacid metabolism	498
tkt	Transketolase	Energy metabolism	459
atpA/uncA	ATP synthase alfa subunit	Energy metabolism	489

The previous allele numbers and ST provide a universal nomenclature that makes MLST results easier to interpret. Furthermore, all the MLST data can be submitted into public data bases (e.g. https://pubmlst.org/) enabling a rapid and accurate comparison among isolates from all over the world (Maiden, 2006).

1.5. Virulence factors

At present the pathogenesis of *Campylobacter* infection is still not well understood. However, it has been seen that not all isolates are equally virulent. Virulence factors are a wide variety of different molecules produced by microorganisms that enable them to establish themselves in the host and enhance its potential to cause disease. Some genes have been recognized as responsible for the expression of putative virulence factors that

may contribute to human campylobacteriosis (Bang et al., 2003) (Datta et al., 2003). These genes are involved mainly in motility (e.g. *flaA* and *flaB*), adherence and colonization of the host (e.g. *cadF, dnaJ, pldA, racR, virB11*), toxin production (e.g. *cdtABC*), cell invasion (e.g. *ceuE* and *ciaB*), expression of Guillain Barré syndrome (*wlaN*) or belong to Type VI secretion system (e.g. *hcp*) (Linton et al., 2000) (Koolman et al., 2015). Since *Campylobacter* may be transmitted from wild animals to livestock and humans it is important to determine their virulence potential.

2. OBJECTIVES

To gain insight into the epidemiology of thermophilic campylobacters, the aim of this study was to characterize *C. coli* and *C. lanienae* isolates from wild boars from Barcelona metropolitan area in terms of genetic diversity using Multilocus Sequence Typing and to assess their virulence potential.

3. MATERIALS AND METHODS

Campylobacter isolates characterized in the present study were recovered from 133 wild boars captured or harvested at three locations on 2015 and 2016: the urban area of Barcelona, the Collserola massif and the campus of the Autonomus Univeristy of Barcelona (UAB). *Campylobacter spp*. isolation was performed using standard culture methods, by steaking the swabs onto selective agar (mCCDA, Oxoid), subculturing onto Columbia blood agar (BioMérieux) to obtain pure cultures and by subsequent PCR identification at species level. Pure cultures were preserved in brain heart infusion with 20% glycerol at -80ºC. A bacterial suspension of each isolate was also performed in 1ml of PBS and was stored at -20ºC for DNA extraction and molecular analyses. A 60% of wild boars sampled carried thermophilic *Campylobacter spp.* The genetic diversity of the isolates had been determined by flaA-RFLP, ERIC-PCR and PFGE.

In this study, a total of 29 *Campylobacter* isolates (21 *C. coli*, 8 *C. lanienae*), representative of the different pulsotypes from the PFGE analyses, were chosen for MLST characterization. In addition, to assess the virulence potential of those isolates, we analyzed the presence of 14 putative virulence genes.

3.1. Genomic DNA extraction

Extraction of total DNA was performed using Instagene™ Matrix (Bio-Rad, Californa, USA): 200µl of bacterial suspension stored in PBS at -80ºC were resuspended in 100 µl of Instagene™ matrix, incubated at 56ºC for 30 min followed by an incubation at 100ºC for 8 min. The extracted DNA was stored at -20ºC.

3.2. MLST

3.2.1. PCR amplification and purification

The primary MLST primer pairs used for the PCR amplification of the seven housekeeping genes of *C. coli* isolates were designed by Miller et al., 2005 (Table 2). The alternative primers used in case of no amplification were those reported by Korczak et al., 2009 (Table 2). For *C. lanienae* isolates, the primary primers designed by Miller et al., 2012 (Table 3) were used, and alternative primers (Miller et al., 2012) were used when no amplicon was obtained with the primary primers.

Each MLST amplification mixture contained: 12,6 µl of Master Mix 2x (Promega, WI, USA), 2 µl of each primer (10 pmol/µl), 6 µl of Nuclease Free water (Promega, WI, USA) and 2,5 µl of genomic DNA. When encountering difficulties to obtain PCR products with either primary or alternative primer sets, an alternative Taq polymerase was used (GoTaq DNA polymerase (Promega, WI, USA). The reaction mixture contained: 5 µl of 5X Buffer, 2 µl of $MgCl_2$ 25mM, 1.25µl of dNTPs 5mM, 2µl of each primer (10pmol/ µl), 9.95 µl of Nuclease free water (Promega, WI, USA), 2,5 µl of genomic DNA and 0.3 units/µl of GoTaq DNA polymerase (Promega, WI, USA).

PCRs were performed in SimpliAmp™ Thermal Cycler (Applied Biosystem®) with different settings depending on the primers used. PCR conditions for both *C. coli* and *C. lanienae* (Miller et al., 2005 and 2012) were the following: 95ºC for 5 min for initial denaturation, followed by 30 cycles of denaturation at 94ºC for 30s, primer annealing at 53ºC for 30s, primer extension step at 72ºC for 2 min and final extension step at 72ºC for 7 min. PCR conditions for the alternative primers by Korckzak et al.(2009), were slightly different: 95ºC for 5 min for initial denaturation, followed by 35 cycles of denaturation at 94ºC for 30s, primer annealing at 56ºC for 1 min, primer extension step at 72ºC for 1 min and final extension step at 72ºC for 7 min.

PCR products were analyzed by electrophoresis on a 1.8% agarose gel. DNA bands were stained with 0.2 µg/ml ethidium bromide and the results were visualized with the UV transilluminator UGENIUS^3 (Syngene, Cambridge, England) and photographed.

Amplicons of MLST were purified using the NucleoSpin® Gel and PCR Clean-up (Macherey- Nagel, Düren, Germany) or the QIAquick 96 well PCR purification kit (Qiagen, Craw-ley, UK), according to manufacturer's instructions. Next, DNA concentration of each isolate was measured with Biodrop. Those samples with more than 15ng/µl were diluted with water as recommended by the sequencing service.

3.2.2. Sequencing of PCR products

The purified PCR products were sequenced using the same primers used for PCR (Table 2 and 3), but at a different concentration (3.2pmol/µl). DNA sequencing was undertaken by Geneservice Source BioScience (Nottingham, United Kingdom).

3.2.3. Sequence analysis

Sanger sequence data was entered, edited and analyzed using Fingerprinting II v3.0 software (Bio-Rad, California, USA). Alleles and sequence types (STs) were assigned based on the MLST scheme provided on the *Campylobacter* PubMLST database (http://pubmlst.org/campylobacter). Novel alleles and STs were submitted to the database.

For *C. lanienae* isolates a maximum likelihood tree based on MLST loci was constructed with MEGA X software.

3.2.4. Virulence-associated genes detection

In total, 20 *C. coli* and 8 *C. lanienae* isolates were tested by PCR for the presence of 14 genes encoding putative virulence factors. The virulence genes testes were related to motility (*flaA* and *flaB*), adhesion and colonization (*cadF*, *dnaJ*, *racR*, *pldA*, *virB11*), invasion (*ceuE*, *ciaB*), cytotoxin production (*cdtA*, cdtB, *cdtC* and *wlaN*) and related with the secretion system T6SS (hcp). GenomicDNA was extracted by standard procedures using the InstaGene matrix Kit (Bio-Rad Laboratories) as indicated above. Primers sets used and the corresponding annealing temperatures are indicated in Table 4. The taq polymerase used was GoTaq DNA polymerase (Promega, WI, USA) and the reaction mixture as described above (3.2.1). PCR conditions were as follows: 5 min at 95ºC for initial denaturation, followed by 35 cycles of denaturation at 94ºC for 30s, annealing at a temperature specific to the primer pair for 1 min (Table 4), and primer extension step at 72ºC for 1 min, and a final extension step at 72ºC for 7 min.

Table 2. *Campylobacter coli* primer sets used for MLST.

Locus	Primer	Forward 5'-3'	Primer	Reverse 3'-5'	Reference
aspA	aspAF1	GAGAGAAAAGCWGAAGAATTTAAAGAT	aspAR1	TTTTTTCATTWGCRSTAATACCATC	Miller et al., 2005
	aspA_Cjc-L	CAACTKCAAGATGCWGTACC	aspA_Cjc-R	ATCWGCTAAAGTATRCATTGC	Korczak et al., 2009
atpA	atpAF	GWCAAGGDGTTATYTGTATWTATGTTGC	atpAR	TTTAADAVYTCAACCATTCTTTGTCC	Miller et al., 2005
	atpA_Cjc-L	CAAAAGCAAAGYACAGTGGC	atpA_Cjc-R	CTACTTGCCTCATCYAAATCAC	Korczak et al., 2009
glnA	glnAF	TGATAGGMACTTGGCAYCATATYAC	glnAR	ARRCTCATATGMACATGCATACCA	Miller et al., 2005
	glnA_Cjc-L	ACWGATATGATAGGAACTTGGC	glnA_Cjc-R	GYTTTGGCATAAAAGTKGCAG	Korczak et al., 2009
gltA	gltAF	GARTGGCTTGCKGAAAAYAARCTTT	gltAR	TATAAACCCTATGYCCAAAGCCCAT	Miller et al., 2005
	gltA_Cjc-L	TATCCTATAGARTGGCTTGC	gltA_Cjc-R	AAGCGCWCCAATACCTGCTG	Korczak et al., 2009
glyA	glyAF	ATTCAGGTTCTCAAGCTAATCAAGG	glyAR	GCTAAATCYGCATCTTTKCCRCTAAA	Miller et al., 2005
	glyA_Cjc-L	AGGTTCTCAAGCTAATCAAGG	glyA_Cjc-R	CATCTTTTCCRCTAAAYTCACG	Korczak et al., 2009
pgm	pgmF1	CATTGCGTGTDGTTTTAGATGTVGC	pgmR1	AATTTTCHGTBCCAGAATAGCGAAA	Miller et al., 2005
	glmM_Cjc-L	GCTTATAAGGTAGCWCCKACTG	glmM_Cjc-R	AATTTTCHGTTCCAGAATAGCG	Korczak et al., 2009
tkt	tktF1	GCAAAYTCAGGMCAYCCAGGTGC	tktR1	TTTTAATHAVHTCTTCRCCCAAAGGT	Miller et al., 2005
	tkt_Cjc-L	AAAYCCMACTTGGCTAAACCG	tkt_Cjc-R	TGACTKCCTTCAAGCTCTCC	Korczak et al., 2009

Table 3. *Campylobacter lanienae* primer sets used for MLST (Miller et al., 2012).

Locus	Primer	Forward 5'-3'	Primer	Reverse 3'-5'
aspA	LANaspF	TTTAGCCACAGCTATGGAGTATCTCAA	LANaspR	ATATGGGTTRAAWGCTGTAACRATACC
	*HFLaspXF	AAYATGAAYGCAAACGAAGTTATAGC	LANaspR	ATATGGGTTRAAWGCTGTAACRATACC
atpA	LANatpF	AACCAAAAAGGTCAAGATGTTATATG	LANatpR	ATTTTCTACTGGAAGTGGGCTATAAGG
glnA	LANglnF	TGGCAYCAYGTATCWTATAATATAAAAGC	LANglnR	ATGGACRTGCATACCRCTWCCATTATC
	*HFLglnXF	TTTYGAATWTTGTRAWGAAAATGAAGT	*HFLglnXF	AGAGTAWGTWAGAATGCTTGGKGCTTC
gltA	LANgltF	ATGCATAGMGGMTATGATATAGCGTGG	LANgltR	CATCAACTCTATCTGGAGTWCCKATCA
glyA	LANglyF	TGCWAATGTTCAGCCAAATAGCG	LANglyR	CAAGAGCGATATCRGCRTCTTTACC
pgm	LANpgmF	GCTTACYTTAAAAGGCCTRMGAGTTGT	LANpgmR	AAGAAGCAGYCTAATCAAATTYTCTGT
tkt	LANtktF	CATCTAAAKCAYAATCCMAAAAATCC	LANtktR	ATCTCWKCGCCAAGMGGAGC

*Alternative MLST primer

Table 4. Virulence factors primers used and annealing temperatures

Locus	Primer	Sequence 5'- 3'	Primer	Sequence 5'- 3'	Annealing temperature (ºC)	Reference
flaA	flaA664	AATAAAAATGCTGATAAAACAGGTG	flaA1494	TACCGAACCAATGTCTGCTCTGATT	55	Datta et al., 2003
flaB	flaB-F	AAGGATTTAAAATGGGTTTTAGAATAAACACC	flaB-R	GCTCATCCATAGCTTTATCTGC	55	Goon et al., 2003
cadF	cadF-F2B	TTGAAGGTAATTTAGATATG	cadF-R1B	CTAATACCTAAAGTTGAAAC	55	Datta et al., 2003
ceuE(Cc)	COL1	ATGAAAAAATATTTAGTTTTTGCA	COL2	GATCTTTTTGTTTTGTGCTGC	55	Bang et al., 2003
racR	racR-25	GATGATCCTGACTTTG	racR-593	TCTCCTATTTTTACCC	40	Datta et al., 2003
dnaJ	dnaJ-299	AAGGCTTTGGCTCATC	dnaJ-1003	CTTTTTGTTCATCGTT	40	Datta et al., 2003
virB11	virB-232	TCTTGTGAGTTGCCTTACCCCTTTT	virB-701	CCTGCGTGTCCTGTGTTATTTACCC	45	Datta et al., 2003
ciaB	ciaB-403	TTTTTATCAGTCCTTA	ciaB-1373	TTTCGGTATCATTAGC	45	Datta et al., 2003
pldA	pldA-84	AAGCTTATGCGTTTTT	pld-981	TATAAGGCTTTCTCCA	45	Datta et al., 2003
cdtA	DS-18	CCTTGTGATGCAAGCAATC	DS-15	ACACTCCATTTGCTTTCTG	55	Hickey et al., 2000
cdtB	cdtB-113	CAGAAAGCAAATGGAGTGTT	cdtB-713	AGCTAAAAGCGGTGGAGTAT	55	Datta et al., 2003
cdtC	cdtC-192	CGATGAGTTAAAACAAAAAGATA	cdtC-351	TTGGCATTATAGAAAATACAGTT	55	Datta et al., 2003
wlaN	wlaN-DL 39	TTAAGAGCAAGATATGAAGGTG	wlaN-DL 41	CCATTTGAATTGATATTTTTG	53	Linton et al., 2000
hcp	hcp-F	CAAGCGGTGCATCTACTGAA	hcp-R	TAAGCTTTGCCCTCTCTCCA	56	Corcionivoschi et al., 2005
	*gltA-F	GCCCAAAGCCCATCATGCACA	*gltA-R	GCGCTTTGGGGTCATGCACA	56	Corcionivoschi et al., 2005

* Internal positive control of the PCR

4. RESULTS

4.1. MLST

A total of 29 isolates (*C. coli* n = 21, *C. lanienae* n = 8) with 22 different pulsotypes were selected for the MLST analysis (Figure 1). For 19 *C. coli* isolates the complete MLST profile was obtained (Table 6), whilst in 2 *C. coli* isolates the ST could not be determined, since some genes could not be PCR-amplified and sequenced (Table 6).

In total, 12 different STs were found among *C. coli* isolates, of which 4 (33%) corresponded to novel STs (ST 9235, ST 9236, ST 9237 and ST 9238). These novel STs were mostly due to new allele combinations (ST 9236, ST 9237 and ST 9238) or to new allele sequences (ST 9235; new allele uncA 588). All *C. coli* isolates with existing ST belonged to the same clonal complex (CC 828). In addition, the novel STs described have at least 4 alleles in common with previously described STs, suggesting that despite this information is currently not available in the pubMLST database, they probably belong to the CC 828 (Table 6).

The most frequent ST among *C. coli* isolates was ST 854 with 5 isolates (SS15068-C1, SS15091-C1, SS15115-C1, SS16005-C2, SS16030-C1). Moreover, this ST was found in isolates from all three studied areas. The second more common ST was 827 with 3 isolates: one from Collserola (SS15185-C1) and 2 isolates from UAB area (SS15078-C1, SS15068-C4). We also found two isolates from different areas (SS15100-C1 from Barcelona and SS15188-C1 from Collserola) with the same novel ST (ST 9235). The remaining 9 STs (ST 828, ST 2814, ST 9237, ST 3020, ST 2097, ST 1058, ST 9238, ST 9236) were found in single isolates and were distributed heterogeneously among the three studied areas (Table 6).

We obtained a higher number of different pulsotypes for *C. coli* isolates (n= 15) than STs (n= 12) (Figure 1). We also detected isolates with different ST, grouped as the same pulsotype (SK3 in ST 854 and ST 828; SK1 in ST 2814 and ST 9237; SK11 in ST 9236 and 9238) and vice versa, isolates with different pulsotypes grouped as the same ST (ST 827 in SK13 and SK14; ST 854 in SK2, SK3, SK6, SK8 and SK9; ST 9235 in SK4 and SK5). (Figure 1).

For *C. lanienae* isolates, the primary primers listed in Table 3, designed by Miller et al. (2012), were able to amplify and sequence successfully all seven loci, except in a few cases in which alternative primers (Miller et al., 2012) had to be used. However, since

all but four alleles (*uncA* 7, *uncA* 12, *glnA* 12, *glyA* 21) obtained in this study were new, we did not submit them to the pubMLST to obtain the corresponding new allele numbers and new STs. To compare *C. lanienae* isolates, a maximum likelihood tree based on concatenated MLST loci was constructed (Figure 2), and the phylogenetic relationships among *C. lanienae* isolates were assessed. Three isolates from two different areas showed 100% similarity (SS15069-C2, Barcelona; SS15127-C1 and SS15190-C1, Collserola) and belonged to the same pulsotype SK16, whilst two other isolates belonging to different pulsotypes were also closely related and differed only in 6 nucleotides (SS15102-C1, SS15132-C2). Isolate SS15102-C4 from Barcelona was the most divergent.

4.2. Comparison with ST in the PubMLST database

The different STs obtained for *C. coli* isolates were compared with global *C. jejuni*/ *C. coli* isolates available in the PubMLST database (Table 7). In our study all obtained *Campylobacter* STs were recovered from wild boars. Most STs according to PubMLST database have been isolated from a wide range of food animals (e.g., sheep's, cattle and pigs) but with more frequency from chickens. Besides the novel STs obtained in the present study, all ST have also been isolated from human stools. Additionally, ST 827, ST 828 and ST 854 have also been isolated from environmental waters.

The main source for ST 827 are human stools which represent a 46.6% of the total number of isolates with this ST. The ST with a higher percentage of chicken isolates is ST 828. In the case of ST 854 and ST 1058 the predominant source are pigs.

4.3. Virulence determinants

We tested the presence of 14 virulence-associated genes in those same *C. coli* and *C. lanienae* isolates analyzed by MLST (Tables 8 and 9).

The highest number of virulence determinants (12) was detected in the following *C. coli* isolates: SS16030-C1 from Collserola (ST 854) and SS15070-C3 from UAB (ST 1058). All but two *C. coli* isolates possessed both motility genes *flaA* and *flaB*, whilst in *C. lanienae* isolates we detected the motility gene *flaB* (100%) but not *flaA*. The genes *ceuE* and *ciaB* involved in invasion were found with different frequency. *CeuE*, a lipoprotein component of a binding-protein-dependent transport system (Bang et al., 2003), was detected in all *C. coli* and *C. lanienae* isolates (100%). On the other hand,

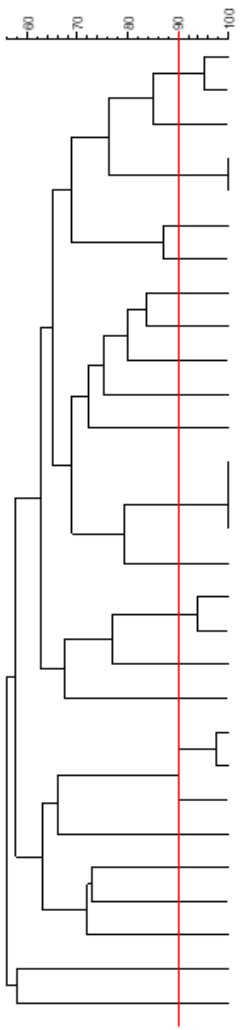
ciaB which encodes a protein required for the internalization of *Campylobacter* by non-professional phagocytic cells (Konkel et al., 1999), was only detected in 25% of *C. coli* isolates and it was not detected at all in *C. lanienae.*

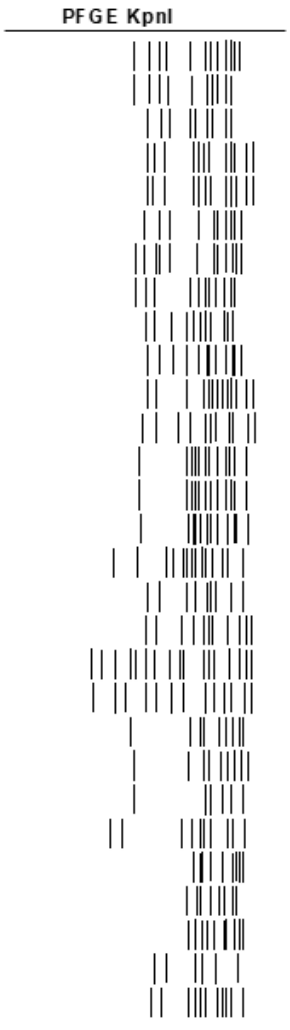
Regarding adherence and colonization genes, their prevalence was unequal: the gene *cadF* which codes for a putative protein that mediates the binding of *Campylobacter* to epithelial cells, was found in 95% of *C. coli* isolates and the gene *virB11*, located in the virulence related plasmid pVir, in 70% of *C. coli* isolates. On the contrary, in *C. lanienae* isolates the prevalence of *cadF* (25%) and *virB11* (25%) was lower. The presence of *racR* (response regulation protein part of the two component regulatory system (TCR)), *dnaJ* (heat shock protein) and *pldA* (phospholipase A), was low in both species.

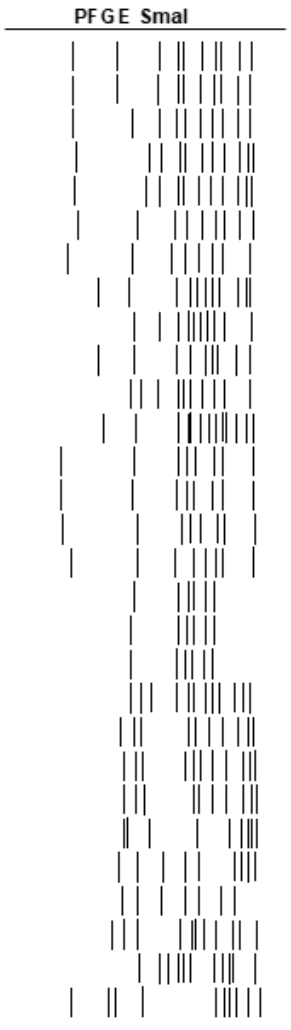
The *hcp* gene coding for a host surface adhesion protein is a component of a type 6 secretion system (T6SS) that has been associated with virulence, was found in all *C. coli* and *C. lanienae* isolates analyzed.

A 65% of *C. coli* isolates carried all three *cdtA*, *cdtB*, and *cdtC* genes, conforming a polycistronic operon, coding for synthesis and deliver of the cytolethal distending toxin (CDT). Most *C. coli* isolates (95%) were positive for some of these genes and only one *C. coli* isolate was PCR-negative for all three *CDT* genes (SS15080-C3). On the contrary, only two *C. lanienae* isolates (25%) were positive for all *CDT* genes.

The only gene that was not detected in any of *C. coli* and *C. lanienae* isolates was *wlaN* gene, which is associated with Guillain-Barré syndrome.







Isolate	Species	Year	Location	Pulsotype	ST	CC
SS16036-C1	*C. coli*	2016	Barcelona	SK1	2814	828
SS16009-C3	*C. coli*	2016	Barcelona	SK1	9237	-
SS16030-C1	*C. coli*	2016	Collserola	SK2	854	828
SS15115-C1	*C. coli*	2015	Barcelona	SK3	854	828
SS15117-C1	*C. coli*	2015	Barcelona	SK3	828	828
SS15188-C1	*C. coli*	2015	Collserola	SK4	9235	-
SS15100-C1	*C. coli*	2015	Barcelona	SK5	9235	-
SS15091-C1	*C. coli*	2015	UAB	SK6	854	828
SS15077-C2	*C. coli*	2015	UAB	SK7	2097	828
SS15068-C1	*C. coli*	2015	UAB	SK8	854	828
SS16005-C2	*C. coli*	2016	UAB	SK9	854	828
SS15118-C1	*C. coli*	2015	Collserola	SK10	3020	828
SS15083-C1	*C. coli*	2015	UAB	SK11	-	-
SS16005-C3	*C. coli*	2016	UAB	SK11	9238	-
SS15080-C3	*C. coli*	2015	UAB	SK11	9236	-
SS15080-C2	*C. coli*	2015	UAB	SK12	-	-
SS15078-C1	*C. coli*	2015	UAB	SK13	827	828
SS15185-C1	*C. coli*	2015	Collserola	SK13	827	828
SS15068-C4	*C. coli*	2015	Barcelona	SK14	827	828
SS15070-C3	*C. coli*	2015	UAB	SK15	1058	828
SS15190-C1	*C. lanienae*	2015	Collserola	SK16	-	-
SS15069-C2	*C. lanienae*	2015	Barcelona	SK16	-	-
SS15127-C1	*C. lanienae*	2015	Collserola	SK16	-	-
SS15102-C4	*C. lanienae*	2015	Barcelona	SK17	-	-
SS15132-C2	*C. lanienae*	2015	Collserola	SK18	-	-
SS15102-C1	*C. lanienae*	2015	Barcelona	SK19	-	-
SS16042-C1	*C. lanienae*	2016	Collserola	SK20	-	-
SS16037-C1	*C. coli*	2016	Collserola	SK21	5006	828
SS16031-C2	*C. lanienae*	2016	Collserola	SK22	-	-

Figure 1. Combined dendogram of SmaI and KpnI PFGE profiles of *C. coli* and C. *lanienae* isolates. The tree was constructed using the UPGMA clustering method and the Dice coefficient for the similarity matrices calculation. Pulsotypes (SK) were assigned with a similarity level ≥ 90%. ST, MLST sequence type; CC, MLST clonal complex. Novel STs are highlighted in red.

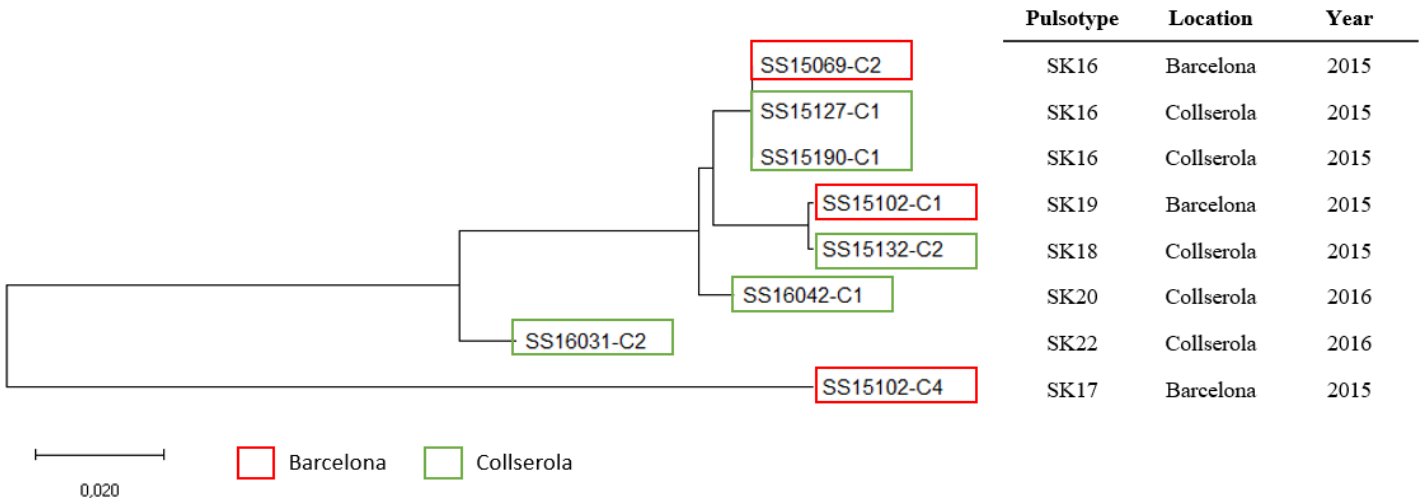
Table 6. MLST allelic profiles and sequence types of *C. coli* isolates. Novel STs and allele numbers are highlighted in red. Isolates are grouped by geographic areas.

Isolate	Year	Location	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*	ST[a]	CC
SS16036-C1	2016	Barcelona	32	38	30	82	104	43	36	2814	828
SS16009-C3	2016	Barcelona	32	38	30	82	104	44	36	9237	-
SS15115-C1	2015	Barcelona	33	38	30	82	104	43	17	854	828
SS15117-C1	2015	Barcelona	33	39	30	82	104	43	17	828	828
SS15100-C1	2015	Barcelona	33	39	44	82	118	35	588	9235	-
SS15068-C4	2015	Barcelona	33	39	30	82	104	56	17	827	828
SS16030-C1	2016	Collserola	33	38	30	82	104	43	17	854	828
SS15188-C1	2015	Collserola	33	39	44	82	118	35	588	9235	-
SS15118-C1	2015	Collserola	33	179	30	79	113	43	17	3020	828
SS15185-C1	2015	Collserola	33	39	30	82	104	56	17	827	828
SS15091-C1	2015	UAB	33	38	30	82	104	43	17	854	828
SS15077-C2	2015	UAB	33	38	30	238	104	43	36	2097	828
SS15068-C1	2015	UAB	33	38	30	82	104	43	17	854	828
SS16005-C2	2016	UAB	33	38	30	82	104	43	17	854	828
SS15083-C1	2015	UAB	-	-	-	-	-	-	-	nd	-
SS16005-C3	2016	UAB	33	39	30	82	118	44	17	9238	-
SS15080-C3	2015	UAB	33	4	30	115	104	85	17	9236	-
SS15080-C2	2015	UAB	33	38	30	115	104	85	-	nd	-
SS15078-C1	2015	UAB	33	39	30	82	104	56	17	827	828
SS15070-C3	2015	UAB	33	39	30	82	104	35	17	1058	828

a) nd, no sequence obtained and thus no allele number could be assigned.

Table 7. ST obtained in this study compared with the wider population of sequence types in the PubMLST database. Highlighted in red the most prevalent source for each ST.

ST	PubMLST isolates	Chicken	Chicken meat	Sheep	Farm environment	Cattle	Pig	Environmental waters	Human stool	Other sources
827	1562	209 (14.7%)	72 (5.8%)	138 (9.7 %)	155 (10.9%)	82 (5.8%)	5 (0.4%)	12 (0.8%)	663 (46.6%)	226 (14.5%)
828	500	298 (64%)	32 (6.9%)	1 (0.21%)	-	4 (0.9%)	27 (5.8%)	6 (1.29%)	70 (15.02%)	62 (12.4%)
854	433	150 (36.5%)	20 (4.87%)	3 (0.7%)	-	8 (2%)	161 (39.2%)	7 (1.7%)	36 (8.8%)	48 (11.1%)
1058	45	2 (4.4%)	5 (11.1%)	-	-	-	31 (68.9%)	-	3 (6.7%)	4 (8.9%)
2097	8	3 (37.5%)	-	-	-	-	-	-	3 (37.5%)	2 (25%)
2814	4	-	-	-	-	-	1 (25%)	-	3 (75%)	-
3020	8	4 (50%)	-	-	-	-	-	-	3 (37.5%)	-
5006	1	-	-	-	-	-	-	-	1 (100%)	-
9235	1	-	-	-	-	-	-	-	-	1 (100%)
9236	1	-	-	-	-	-	-	-	-	1 (100%)
9237	1	-	-	-	-	-	-	-	-	1 (100%)
9238	1	-	-	-	-	-	-	-	-	1 (100%)



Pulsotype	Location	Year
SK16	Barcelona	2015
SK16	Collserola	2015
SK16	Collserola	2015
SK19	Barcelona	2015
SK18	Collserola	2015
SK20	Collserola	2016
SK22	Collserola	2016
SK17	Barcelona	2015

Figure 2. Maximum likelihood tree based on concatenated MLST loci of *C. lanienae* isolates. Branches lenghts are measured in the number of substitutions per site.

Table 8. Presence of virulence associated genes depicted as blue squares**.**

			Virulence genes														
			Motility		Adhesion and colonization					T6SS	Invasion		Toxin production				
Isolate	**Species**	**ST**	***flaA***	***flaB***	***cadF***	***racR***	***dnaJ***	***pldA***	***virB11***	***hcp***	***ceuE***	***ciaB***	***cdtA***	***cdtB***	***cdtC***	***wlaN***
SS16036-C1	*C. coli*	2814	■	■	■					■	■		■	■		
SS16009-C3	*C. coli*	9237	■	■	■		■			■	■		■	■	■	
SS16030-C1	*C. coli*	854	■	■	■	■		■	■	■	■	■	■	■	■	
SS15115-C1	*C. coli*	854	■	■	■				■	■	■			■		
SS15117-C1	*C. coli*	828	■	■	■		■		■	■	■		■	■	■	
SS15188-C1	*C. coli*	9235	■	■	■				■	■	■	■		■		
SS15100-C1	*C. coli*	9235	■	■	■				■	■	■			■		
SS15091-C1	*C. coli*	854	■	■	■					■	■	■	■	■	■	
SS15077-C2	*C. coli*	2097	■	■	■		■		■	■	■		■	■	■	
SS15068-C1	*C. coli*	854	■	■	■				■	■	■		■	■	■	
SS16005-C2	*C. coli*	854	■	■	■				■	■	■			■		
SS15118-C1	*C. coli*	3020	■	■	■					■	■		■	■	■	
SS16005-C3	*C. coli*	9238	■		■				■	■	■	■	■	■	■	
SS15080-C3	*C. coli*	9236	■	■	■					■	■					
SS15080-C2	*C. coli*	-		■	■			■	■	■	■	■	■	■		
SS15078-C1	*C. coli*	827	■	■	■		■		■	■	■		■	■	■	
SS15185-C1	*C. coli*	827	■	■						■	■			■		
SS15068-C4	*C. coli*	827	■	■	■	■	■	■		■	■		■	■	■	
SS15070-C3	*C. coli*	1058	■	■	■	■	■	■	■	■	■		■	■	■	
SS16037-C1	*C. coli*	5006	■	■	■	■	■	■		■	■		■	■	■	
SS15190-C1	*C. lanienae*	-		■	■	■	■			■	■		■	■	■	
SS15069-C2	*C. lanienae*	-		■	■	■	■	■		■	■		■	■	■	
SS15127-C1	*C. lanienae*	-		■				■		■	■					
SS15102-C4	*C. lanienae*	-		■					■	■	■					
SS15132-C2	*C. lanienae*	-		■			■	■		■						
SS15102-C1	*C. lanienae*	-		■				■		■	■					
SS16042-C1	*C. lanienae*	-		■						■	■			■		
SS16031-C2	*C. lanienae*	-		■					■	■	■					

Table 9. Prevalence (%) of virulence genes among *C. coli* and *C. lanienae* isolates.

Loci	*C. coli (n=20)*	*C. lanienae (n=8)*
flaA	19 (95)	0 (0)
flaB	19 (95)	8 (100)
cadF	19 (95)	2 (25)
racR	4 (20)	2 (25)
dnaJ	7 (35)	3 (37.5)
pldA	5 (25)	4 (50)
virB11	14 (70)	2 (25)
hcp	20 (100)	8 (100)
ceuE	20 (100)	7 (87.5)
ciaB	5 (25)	0 (0)
cdtA	14 (70)	2 (25)
cdtB	19 (95)	3 (37.5)
cdtC	12 (60)	2 (25)
wlaN	0 (0)	0 (0)

5. DISSCUSSION

Campylobacter spp., have been identified as the most common cause of human gastroenteritis worldwide. Up to now many efforts have been taken to reduce human incidence of campylobacteriosis, nevertheless, the cases are still increasing (EFSA, 2017) (WHO, 2012). To control this zoonosis is necessary to better understand the global epidemiology of the pathogen, its reservoirs and the pathogenicity of the different strains. Furthermore, special emphasis should be made on wild animals, since they constitute another reservoir for *Campylobacter spp.* In this study we investigated the genetic diversity and virulence potential of *C. coli* and *C. lanienae* isolates from wild boars of the metropolitan area of Barcelona.

Campylobacter isolates from wild boars had been previously characterized by PFGE and those *C. coli* and *C. lanienae* isolates from representative pulsotypes were typed with MLST. However, for two isolates the complete MLST profile could not be obtained, as in one case one allele was amplified but could not be sequenced (SS15080-C2) and in the other case (SS15083-C1) no amplification was achieved with any of the MLST genes. One possible explanation for this lack of amplification of the isolate SS15083-C1 could be a low concentration or bad quality of DNA in the initial bacterial suspension. Another explanation might be that the primers used could not hybridize with the DNA sequence of the isolate due to the possible highly genetic variability of

this isolate. These problems highlight some of the disadvantages of MLST which is still time consuming as for problematic isolates, reactions have to be repeated several times in order to obtain all seven loci needed for ST determination.

Both typing methods (PFGE and MLST) showed a high genetic diversity of *C. coli* isolates since 15 pulsotypes (PFGE) and 12 ST were identified. *C. lanienae* isolates were also genetically diverse, as shown with the maximum likelihood tree and the 7 profiles identified by PFGE. Differences between the results obtained with PFGE and MLST may be explained by the different discriminatory level of both techniques and the high genetic instability of *Campylobacter* genome. In some isolates same pulsotypes turned to be different ST, probably as a result of single nucleotide changes that do not alter the restriction sites and therefore are not reflected in band profiles (pulsotyes). *Campylobacter spp*. has been identified to undergo frequent DNA changes in response to different environments and hosts to increase their potential of adaptation to new situations (Dingle et al., 2002) (Miller et al., 2006). This diversity across the MLST loci is important for the potential identification of alleles or ST that may correlate with animal host or geographical location (Miller et al., 2006).

In this study we described 4 new ST of *C. coli* and 1 new allele. Novel MLST data was submitted to the corresponding PubMLST, contributing to the understanding of *C. coli* epidemiology. Among *C. coli* isolates MLST analysis showed a predominant ST (ST 854). This ST have been found to be over represented in pigs but it has also been isolated from chicken, livestock and environmental waters (Sheppard et al., 2009). All existing STs described in this study have been associated with human disease and some of them (ST827, ST828, ST854, ST1058) have a worldwide distribution, thus highlighting to the role wild boars can potentially play in human infection.

Despite the high diversity of ST described among *C. coli* isolates, all belonged to the same CC (CC-828), indicating a close relationship among them. The high prevalence of the CC 828 was consistent with the prevalence of this CC found in other studies (Sheppard et al., 2009). In a broad study performed in broiler farms from Catalonia this lineage was also found in all *C. coli* isolates (Cantero, 2017). It should be noted that contrary to *C. jejuni* that is more diverse genetically, few *C. coli* CC have been described to date and most isolates belong to this CC-828.

Certain *C. coli* ST and *C. lanienae* genotypes were detected along the sampled years in the different studied areas, suggesting a possible circulation of the strains as a consequence of the mobility of wild boars between urban and rural areas.

In the case of *C. lanienae* no coincidences were found among alleles of this study and those published in the pubMLST database. The *C. lanienae* MLST database has only 171 different STs whereas *C. coli*/ *C. jejuni* database has 9238 STs. The small number of isolates in the pubMLST database can partially be explained by the difficulties to isolate this species, as in most cases conventional culturing methods are not successful (Schweitzer et al., 2011). In previous reports *C. lanienae* had been isolated mainly from pigs and wild boars' feces and from other wild ruminants. The role of this species in foodborne disease was unknown until 2016, when it was reported the first case of campylobacteriosis caused by *C. lanienae*, showing the potential of this species to cause human diarrheal disease (Lévesque et al., 2016).

We hypothesize that because of the feeding habits of wild boars in the studied areas, they became infected with *Campylobacter* strains of anthropogenic origin (human and food animals). These animals frequently feed from rubbish in urban areas like Barcelona. These infected wild boars represent a potential public health threat since wild boar populations are expanding and increasing their presence in urban areas. Although the overall influence of wild boars on human campylobacteriosis is likely to be small, hunters may be at high risk of exposure during the handling and processing of wild boar meat. Moreover, wild boars while feeding or defecating in fields and crops may contaminate fruits and vegetables with *Campylobacter* (Jay-Russell et al., 2012). Besides humans, wild boars could transmit these foodborne pathogens to domestic or free-ranging animals.

The present study also provides information about the virulence potential of *C. coli* and *C. lanienae* isolates. For colonization to take place in the intestine, it is required that the bacteria have the capacity of motility, adhesion to intestinal mucosa, invasion and production of toxins (Bang et al., 2003). On average, virulence genes were less prevalent in *C. lanienae* isolates compared with *C. coli* isolates. The primers used in both species for PCR amplification of virulence factors were designed for *C. jejuni* and *C. coli*. The specificity of primers along with possible significant differences among *C. lanienae* alleles compared with *C. coli* alleles could explain the lack of amplification of

some virulence genes in *C. lanienae* isolates, rather than their absence in the studied isolates. Among the flagellar genes, *flaB* was present in both *Campylobacter* species, whilst *flaA* was only present in *C. coli* isolates. Flagellar genes are important for *Campylobacter* motility which is essential for intestine colonization, as the flagellar filament helps the bacteria to resist gut peristalsis and survive in the hostile environment of the stomach (Koolman et al., 2015). Genes involved in adhesion (*cadF, racR, dnaJ*) and invasion (*ceuE, virB11, ciaB* and *pldA*) were less conserved and presented a heterogeneous distribution within *C. coli* and C. *lanienae* isolates as previously reported by Datta et al. (2003).

In contrast with other studies, the prevalence of *virB11* in *C. coli* isolates was high (70%) (Koolman et al., 2015). This gene encodes a putative type IV secretion system, a mutation of which can reduce the adherence of the bacteria in the intestinal tract, resulting in a significant reduced virulence (Bacon et al., 2000). The *wlaN* gene, which encodes for a protein involved in cell wall synthesis, responsible for the appearance of Guillain-Barré syndrome, was not detected in any isolate. The absence or low prevalence of this gene in many *Campylobacter* strains has been observed in other studies (Koolman et al., 2015) (Datta, Niwa and Itoh, 2003). The gene *hcp* is a component of the type VI secretion system (T6SS) and it is associated with more severe forms of campylobacteriosis as it can confer cytotoxicity towards red blood cells. Moreover, *hcp*+ strains have increased abilities to adhere and invade the host intestine, conferring more virulence to strains. The gene *hcp* was found in all *C. coli* and *C. lanienae* isolates analyzed. This high prevalence among *C. coli* isolates has previously been reported (Corcionivoschi et al., 2015), whilst it is the first report of the presence of a T6SS in *C. lanienae*.

Cytolethal distending toxin genes (*cdtABC*) had a high prevalence among *C. coli* isolates as previously reported (Koolman et al., 2015). While *cdtB* was detected in all *C. coli* isolates, *cdtA* and *cdtC* were detected at a lower frequency which is in agreement with previous studies (Koolman et al., 2015) (Bang et al., 2003). *CdtA* and *cdtC* are necessary for binding to the host cell, while *cdtB* is the active subunit that enters to the cell and causes cell death. Bang et al. (2003) demonstrated that isolates without either *cdtA* or *cdtC* genes produced little or no CDT.

No relation was found between virulence genes and STs as isolates with the same sequence type presented different combinations of virulence genes. Also, no association between the different studied areas and virulence-associated factors was found.

6. CONLCUSIONS

PFGE and MLST have demonstrated their usefulness in order to study the genetic diversity and population structure of *Campylobacter* strains in wild boars, providing insight into the epidemiology of this zoonotic agent.

Our results also highlight that wild boars from the metropolitan area of Barcelona can act as carriers of *Campylobacter* genotypes associated with human gastroenteritis, some of which have a high virulence potential. These results are of public health concern since contact between humans and wild boars is becoming more common due to changes in their habits and ecological traits. It seems likely that wild boars may have acquired these pathogens due to their close contact with humans in the studied area, although other wild animals or livestock cannot be ruled out. Therefore, wild boars play an important role in the dispersion and in the global epidemiology of *Campylobacter* spp., which represents a challenge for wildlife management.

Further research into the molecular epidemiology of campylobacteriosis combining *Campylobacter* spp. in wild animals and human clinical cases will improve our understanding of the transmission pathways and the dynamics of the disease in humans, which is still not clear.

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