Deliverable D-JRP15–FED-AMR-WP3.2: Overview of genetic overlap between human and non-human *Clostridiodes difficile* isolates

Workpackage 3

Responsible Partner: 36-INSA

Contributing partners: 2-AGES, 9-BfR, 10-FLI, 13-SSI, 14-UT, 20-IP

GENERAL INFORMATION

European Joint Programme full title	**Promoting One Health in Europe through joint actions on foodborne zoonoses, antimicrobial resistance and emerging microbiological hazards**
European Joint Programme acronym	**One Health EJP**
Funding	**This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830.**
Grant Agreement	**Grant agreement n° 773830**
Start Date	**01/01/2018**
Duration	**60 Months**

DOCUMENT MANAGEMENT

Title OHEJP deliverable	**D-JRP15-FED-AMR-WP3.2**
WP and task	WP3, JRP15-WP3-Task 3
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Due month of the deliverable	M60
Actual submission month	M60
Type *R: Document, report* *DEC: Websites, patent filings, videos, etc.; OTHER*	R, DEC, other **Save date**:
Dissemination level *PU: Public (default)* *CO: confidential, only for members of the consortium (including the Commission Services).*	PU *See updated Grant Agreement*
Dissemination *Author's suggestion to inform the following possible interested parties.*	OHEJP WP 1 ☐ OHEJP WP 2 ☐ OHEJP WP 3 ☒ OHEJP WP 4 ☐ OHEJP WP 5 ☐ OHEJP WP 6 ☐ OHEJP WP 7 ☐ Project Management Team ☒ Communication Team ☒ Scientific Steering Board ☒ National Stakeholders/Program Owners Committee ☐ EFSA ☐ ECDC ☐ EEA ☐ EMA ☐ FAO ☐ WHO ☐ OIE ☐ Other international stakeholder(s): …… Social Media: ……

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D-JRP15-FED-AMR-WP3.2

WP3-T3 Evaluation of the extent of genetic overlap between human and non-human *Clostridiodes difficile* lineages

Background

Since its first discovery, *Clostridioides diffcile* has been recognized as the number one cause of hospital acquired antibiotic associated diarrhoea, with severe infections developing into pseudomembranous colitis (1). Symptomatic infections usually arise from a disturbance of the gastrointestinal microbiota following antimicrobial administration and are due to the production of the bacterium's main virulence factors, enterotoxins A and B (2,3). Epidemiologic surveillance programs show that *C. difficile* infections (CDI) are trending from a nosocomial nature towards a more community acquired infection (4).
With a growing awareness in regard to this reality and a clearer case definition, several studies have been reporting concerning rates of community-acquired *C. difficile* infection (CA-CDI) that can be as high as 41% (5). A rising number of studies report the isolation of *C. difficile* in food producing animal faeces (6–8), abattoir samples (9), food products (10) and environmental samples (11), reinforcing the importance of understanding the potential role assumed by these and other non-hospitalar reservoirs in the epidemiology of *C. difficile* infection. In addition, antimicrobial resistance (AMR) is frequently reported in epidemic *C. difficile* strains and is thought to play a major role in the infection and dissemination of this pathogen. *C difficile* has also been suggested as a reservoir/receptor of resistance genes that might be transferred to other species in the host gut as well as in the environment. Currently, there is a gap in knowledge regarding the true impact of *C. difficile* as a zoonotic agent and its transmission networks, including AMR dissemination.

Description of the task

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Task Participants: 2-AGES, 9-BfR, 10-FLI, 14-UT, 20-IP, 36-INSA

This task aims to contribute to increase knowledge regarding the true impact of *C. difficile* as a zoonotic agent and its transmission networks, by evaluating the extent of genetic overlap and potential transmission between human and non-human *C. difficile* lineages. Whole-genome sequencing data from strains isolated from different sources were analysed in order to: i) infer the phylogenetic relationship between strains, through the alignment of genomes and extraction of core single-nucleotide variant positions, ii) describe the general trends of the core-genome determined within the dataset, and iii) identification of mobile genetic elements.

In line with this, within WP3-T3, several studies were conducted with that purpose, involving several sampling campaigns to obtain isolates from animals, food and environment, focusing on relevant zoonotic *C. difficile* lineages.

Description of deliverable

1. RT078/106 (ST 11) genetic analysis

ST11 is considered one of the most pathogenic *C. difficile* types and a common type identified in many international studies, both from clinical and veterinary environments. In Denmark, it is the second most common clinical type and the most common type with the binary toxin and we therefore have a special interest in understanding the transmission and epidemiology in and between different environments, in addition to its antimicrobial resistance and participation in the exchange of resistance genes (Figure 1).

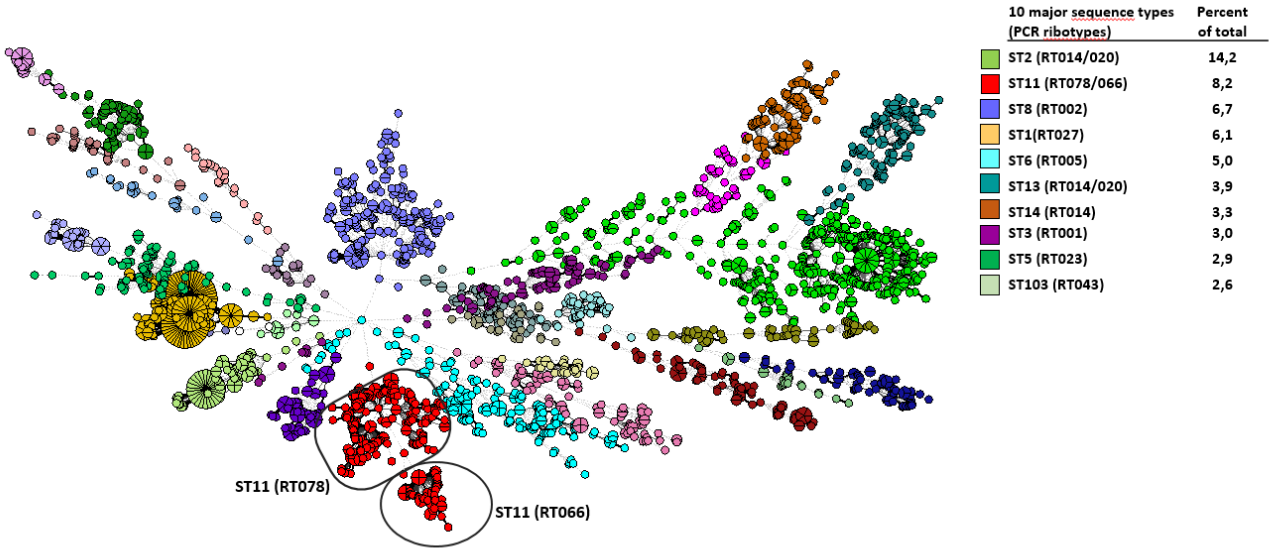


Figure 1. Population structure obtained by minimum spanning tree of core-genome MLST (cgMLST) (BioNumerics, 1999 alleles) of major Danish clinical *C. difficile* sequence types (STs) (colored) derived from WGS data on 2788 isolates obtained from the national sentinel surveillance 2018-2022 (all toxigenic isolates collected one month in spring and fall from all Danish Dept. of clinical Microbiology). Two different ST11, i.e. RT078 and RT066 are circled on figure.

In order to investigate this further, we collected isolates from both clinical and veterinary environments and performed WGS. WGS data was compared by cg/wg-MLST and antimicrobial resistance genes (ARG) and mobile genetic elements (MGE) were investigated by AMRFinder and an MGE tool developed in this consortium. First, investigating only strains from Denmark (479 clinical and 21 porcine fecal farm samples), we found three cg/wgMLST clusters (two from RT078 and one from RT066) containing both human and porcine isolates with genetic distances within possible transmission distances (Figure 2 and Table 1).

This meeting is part of the European Joint Programme One Health EJP.
This project has received funding from the European Union's Horizon 2020
research and innovation programme under Grant Agreement No 773830.

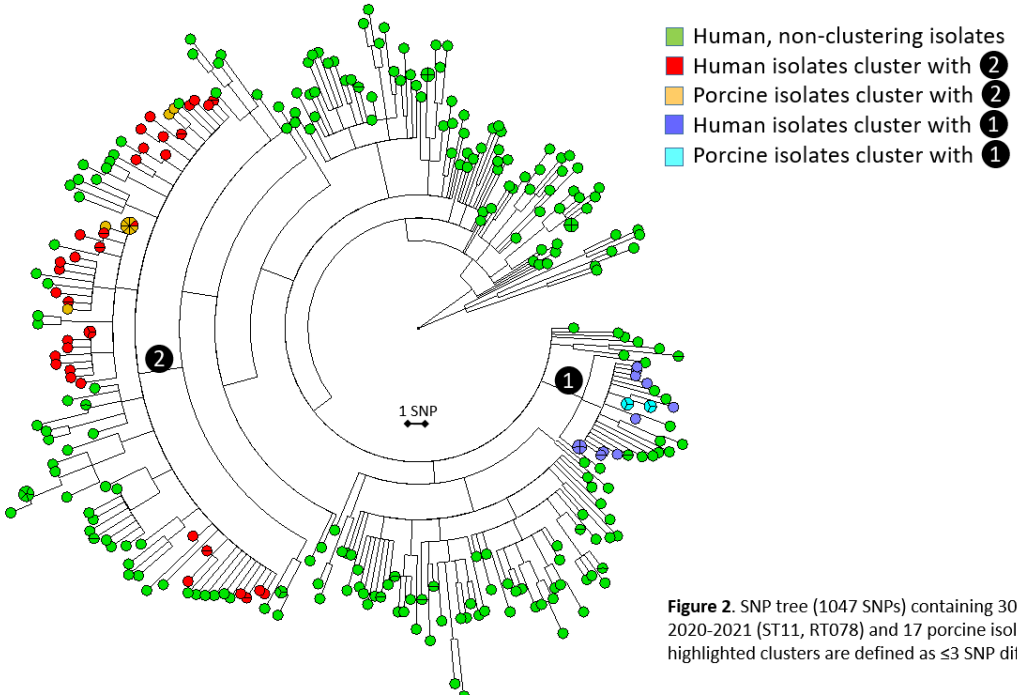


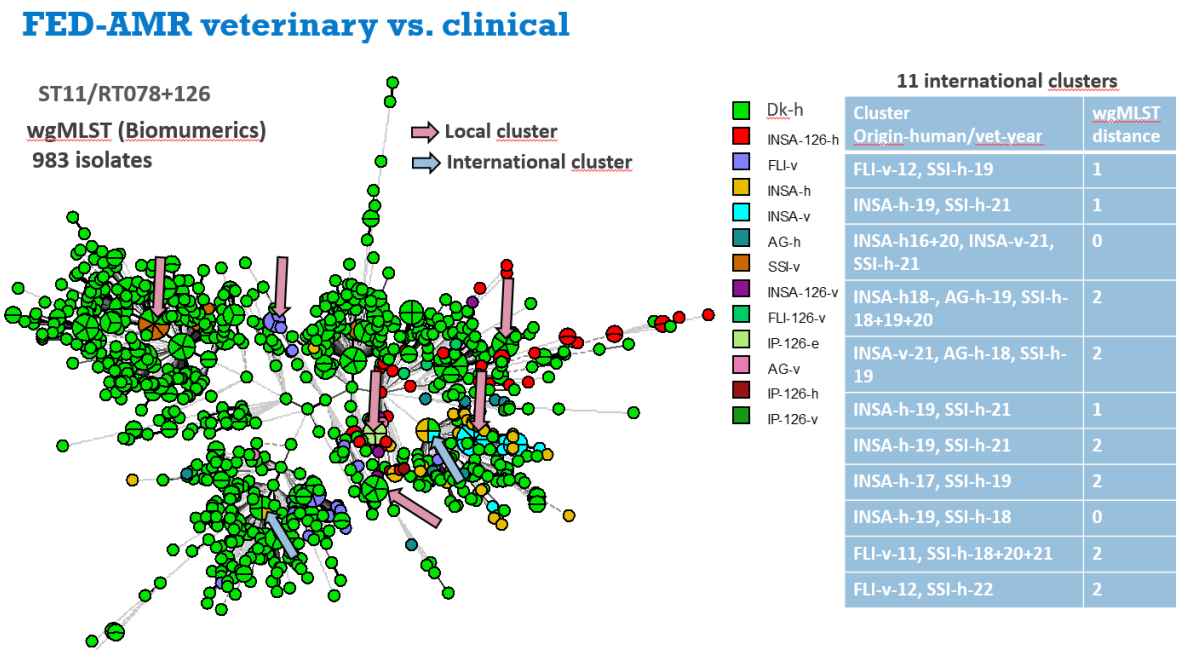
Figure 2. SNP tree (1047 SNPs) containing 308 clinical isolates from 2020-2021 (ST11, RT078) and 17 porcine isolates (2020). The two highlighted clusters are defined as ≤3 SNP differences.

Table 1. Characteristics of the three different ST11 veterinary-human clusters. Cluster 1 and 2 of ST11(RT078) are shown on Figure 2.

Cluster # (ST/RT)	No. porcine isolates (Origin)	No. human isolates (% of total human)	Major ARGs
1 (Figure 2) (ST11/RT078)	6 (Farm #1, 2)	14 (4.5%)	β-lactam: *blaCDD* Fluoroquinolone: *gyrB* (S366V+S416A)
2 (Figure 2) (ST11/RT078)	11 (Farm #2, 6, 8, 9, 10)	35 (11.4%)	β-lactam: *blaCDD* Fluoroquinolone: *gyrB* (S366V+S416A) Aminoglycoside: *ant(6), aph(3), sat4* Tetracycline: *tet(M)*
3 (ST11/RT066)	1 (Farm #8)	12 (14%)	β-lactam: *blaCDD* Fluoroquinolone: *gyrB* (S366V+S416A)

Secondly, we analyzed both human and veterinary strains from FED-AMR partners (Austria (n=12); France (n=7); Germany (n=32), Portugal (n=86) and Denmark (n=1132)). Here, we found that most clusters were among strains from the same country, here again, underlining the finding that human and veterinary strains cluster within possible transmission range. Additionally, we identified 11 international clusters, where strains from two or three different countries clustered within possible transmission range, emphasizing that this clone may have a transmission network that extends beyond country borders (Figure 3). ARG analysis found that the majority of strains were genetically resistant towards aminoglycoside, beta-lactamase, fluoroquinolone and tetracyclin and tetracycline was exclusively associated with transposons. ARGs towards steptothricin and macrolide were found in 67% and 16% of the strains, respectively, macrolide ARG being transposon associated. In conclusion, the genetic overlap found from different environments, makes it plausible that ST11 is transmitted between those, both locally and internationally. The many important ARGs, some on mobile genetic elements, makes it

an important threat to the environments in where it persists, both as a hard-to-eliminate bacteria and as a possible contributor to resistance in other bacteria in the shared environment.
These results will be part of a manuscript in preparation: *Semeh Bejaoui, Jesper Nielsen, Monica Oleastro, Christian Seyboldt, Sven Maurischat, Adriana Cabal Rosel, Christelle Mazuet, Dorte Frees and Søren Persson (+NN to be determined later). Clostridioides difficile (ST11). Time resolved phylogeny and genetic overlap between human and non-human isolates across Europe.*



11 international clusters

Cluster Origin-human/vet-year	wgMLST distance
FLI-v-12, SSI-h-19	1
INSA-h-19, SSI-h-21	1
INSA-h16+20, INSA-v-21, SSI-h-21	0
INSA-h18-, AG-h-19, SSI-h-18+19+20	2
INSA-v-21, AG-h-18, SSI-h-19	2
INSA-h-19, SSI-h-21	1
INSA-h-19, SSI-h-21	2
INSA-h-17, SSI-h-19	2
INSA-h-19, SSI-h-18	0
FLI-v-11, SSI-h-18+20+21	2
FLI-v-12, SSI-h-22	2

Figure 3. SNP tree containing 1269 *Clostridioides difficile* clinical isolates from ST11/RT078, from both human (h) and animal (v) origin. The highlighted clusters are defined as ≤2 SNP differences.

2. RT106 and RT014/020 genetic analysis

Clostridioides difficile infection is the number one cause of antibiotic associated diarrhoea historically involved in nosocomial outbreaks in humans. Recent data reveals an epidemiological shift towards a community acquired infection, raising questions about transmission dynamics of this pathogen. Studies have focused on assessing *C. difficile* zoonotic potential and the role of environmental reservoirs in the community transmission network. While some studies seem to support the foodborne route, this is unlikely to be the sole route accountable for all intercommunity spread. The need to identify additional non-human reservoirs led researchers to turn to companion animals as a possible reservoir of toxigenic strains. This study contributes to clarifying the epidemiological role of companion animals in the *C. difficile* panorama and adds valuable information regarding the genetic overlap between pets and humans' *C. difficile* isolates. The results presented herein support the possibility of interspecies transmission or of a shared environmental contamination source affecting both species. With the number of companion animal owing households expected to increase in coming years, research like this is of paramount importance to determine any potential public health risks, guide health authorities in outlining efficient mitigation measures and increase public awareness of appropriate hygiene practices.

Material and Methods

Animal samples collection

The 475 faecal samples from dogs and cats included in this study were collected by means of convenience sampling and grouped considering the sampling context. One group of animals (group A, n=292) was sampled by veterinary professionals at two veterinary hospitals in Portugal, located at the two biggest and most populated Portuguese urban centres, between July and August 2021. Stool samples were obtained either by rectal swab or by digital rectal collection depending on animal size, and each sample was accompanied by a questionnaire briefly covering the recent clinical history (antibiotic administration and stool consistency), demographic data and environmental living conditions of each animal. The other group of samples (group B, n=183) was provided by a veterinary diagnostic laboratory which receives samples from several Portuguese veterinary hospitals and clinics.

Isolation of Clostridioides difficile

Around 0.5 g of each stool sample was enriched in 5 ml of *C. difficile* enrichment broth for a week under anaerobic conditions, generated using the anaerobic cultivation system Anoxomat (Anoxomat, Mart), at 37◦C. For rectal swabs, these were directly inoculated in 5 mL of C. difficile enrichment broth. Following this step, all samples were subjected to ethanol shock before inoculating the resulting pellet onto ChromID® C. difficile agar for 48-72 hours under anaerobic conditions at 37◦C.

Toxin profile, ribotyping and antimicrobial resistance

Each sample was assessed for the presence of C. difficile based on colony morphology. After species confirmation by MALDI-TOF, genomic DNA was extracted using the Isolate II Genomic DNA kit. Each isolate was characterized by multiplex PCR, targeting gluD and the tcdA, tcdB, cdtA and cdtB toxin genes, and by PCR-ribotyping using Bidet primers followed by capillary gel-based electrophoresis. Antimicrobial susceptibility was performed by disc diffusion for: moxifloxacin (5 µg, ≥20 mm), vancomycin (5 µg, ≥19 mm), metronidazole (5 µg, ≥23 mm) and rifampicin (5 µg, ≥20 mm). For clindamycin the Etest® strips were used and strains were categorized according to the Clinical & Laboratory Standards Institute breakpoint (≥8 mg/L).

Whole genome sequencing and assembly

For the present study, 83 *C. difficile* isolates from Portugal, belonging to contemporary human CDI cases (n = 41), canines (n = 33) and felines (n = 9) from the present study, all belonging to the main toxinogenic types found, RT014/RT020 and RT106, were considered for deeper genetic analysis by whole genome sequencing (WGS). DNA was subjected to Nextera XT library preparation (Illumina, San Diego, CA,

USA) prior to paired-end sequencing (2×250 bp or 2×150 bp) on either a MiSeq, NextSeq 550 or NextSeq 2000 instrument (Illumina), according to the manufacturer's instructions.

Comparative genomic analysis and phylogeny

In order to compare the genome background of *C. difficile* isolates collected from human infections and companion animals, RT014/R020 and RT106 quality-processed reads were mapped against reference genomes. Core-single nucleotide polymorphism (SNP) were extracted using Snippy's core module (snippy-core) ensuring that all genomes reached at least 70% of aligned bases with the reference (which occurred for all sequenced samples). Minimum spanning trees (MST) were constructed using GrapeTree. Genetic clusters with potential epidemiological relevance, i.e., as potential short-term transmission, were defined at a SNP distance threshold of ≤ 2, as previously reported.

Results

Population characteristics and *Clostridioides difficile* positivity rate

Data regarding the positivity rate by variable is summarized in Table 2:

Table 2. Population characteristics and *Clostridioides difficile* positivity rate in groups A and B.

Variable	Parameters	n (%)	Positive n (%)
Group A (Veterinary Hospitals)			
Species	Canine	235 (80.5%)	64 (27.2%)
	Feline	57 (19.5%)	12 (21.1%)
Sex	Male	152 (52.1%)	35 (23%)
	Female	140 (47.9%)	41 (29.3%)
Age	<1	36 (12.3%)	8 (22.2%)
	1-8	143 (49%)	40 (28%)
	>8	113 (38.7%)	28 (24.8%)
Diarrhoea	Yes	60 (20.6%)	22 (36.7%)
	No	229 (78.4%)	52 (22.7%)
	Unknown	3 (1%)	2 (66.7%)
ATB	Yes	9 (3.1%)	2 (22.2%)
	No	161 (55.1%)	47 (29.2%)
	Unknown	122 (41.8%)	27 (22.1%)
Group B (Veterinary Diagnostic Laboratory)			
Species	Canine	100 (54.6%)	23 (23%)
	Feline	83 (45.4%)	11 (13.3%)
Sex	Male	85 (46.4%)	19 (22.4%)
	Female	78 (42.6%)	15 (19.2%)
	Unknown	20 (10.9%)	-
Age	<1	54 (29.5%)	6 (11.1%)
	1-8	83 (45.4%)	20 (24.1%)
	>8	20 (10.9%)	3 (15%)
	Unknown	26 (14.2%)	4 (15.4%)

The distribution of RTs is summarized in Figure 4.

Overall, considering the sum of the isolates from the two groups (n=129), the two toxigenic RTs most commonly found were RT106 (24.8%) and RT014/020 (11.6%).

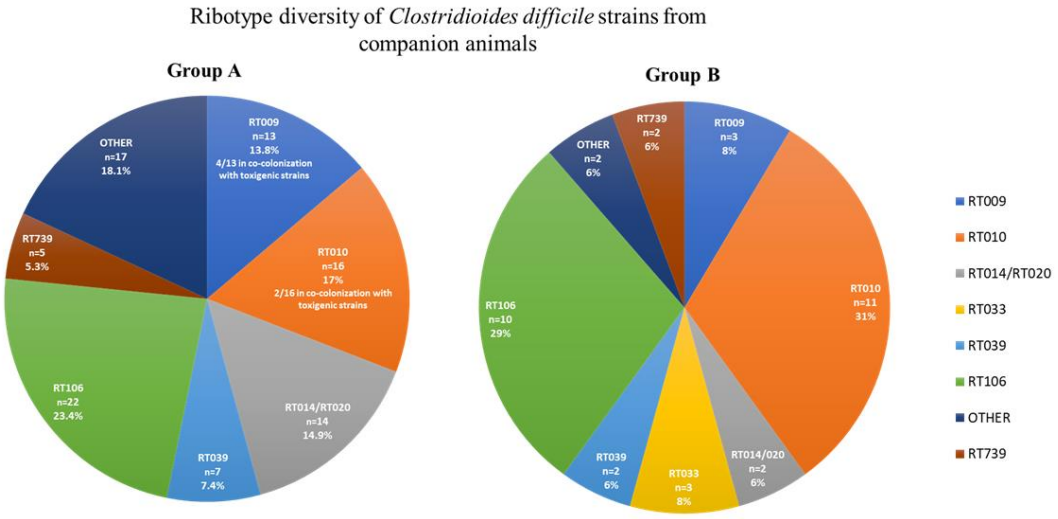


Figure 4: Ribotype diversity of *Clostridioides difficile* strains isolated from companion animals in group A (veterinary hospitals, n=94) and group B (veterinary diagnostic laboratory, n=35). Other = all other ribotypes with prevalence < 5%.

Antimicrobial resistance

In group A (Table 3), the highest rate of resistance was observed for clindamycin, 25.5% (24/94), moxifloxacin and metronidazole, with 13.8% (13/94) and 12.8% (12/94), respectively. Resistance to metronidazole and clindamycin was mainly found in RT010, while moxifloxacin resistance was predominantly found in RT106.

In group B (Table 3), the highest resistance rate was detected for clindamycin, 34.3% (12/35), followed by metronidazole, 28.6% (10/35). Resistance to moxifloxacin was found in 8.6% (3/35) of the *C. difficile* isolates. Regarding combined resistance, 20% (7/35) of the isolates were resistant to metronidazole and clindamycin and 2.9% (1/35) were resistant to moxifloxacin and clindamycin. A total of 5.7% (2/35) of the isolates were MDR. Similarly to group A, resistance to metronidazole was exclusively found in RT010 isolates, while clindamycin resistance showed a wider distribution among RTs but were still mainly represented by RT010 isolates.

Table 3. Antimicrobial resistance prevalence and genetic determinants of resistance in groups A and B.

Resistant Phenotype	Group A % (n/N)	Group B % (n/N)	Total % (n/N)	AMR genetic determinants * (n isolates)	Main RT associated (n isolates)
Clindamycin	25.5% (24/94)	34.3% (12/35)	27.9% (36/129)	*ermB* (36)	RT010 (23)
Moxifloxacin	13.8% (13/94)	8.6% (3/35)	12.4% (16/129)	*gyrA* Thr82Ile (16)	RT106 (8)
Metronidazole	12.8% (12/94)	28.6% (10/35)	17.1% (22/129)	pCD-METRO plasmid (22)	RT010 (22)
Rifampicin	2.1% (2/94)	-	2.1% (2/94)	*rpoB* Arg505Lys (1) *rpoB* His502Asn and Arg505Lys (1)	none

* Determined by PCR and/or Sanger sequencing. The *gyrA* mutation in RT106 isolates was confirmed by WGS.

Genetic diversity of *Clostridioides difficile* RT106 and RT014/020 isolates collected in Portugal

Forty-two *C. difficile* RT106 isolates collected in Portugal, isolated from companion animals and humans, were subjected to WGS. The selected genomes were integrated with previously sequenced RT106 genomes (n=43) from distinct countries (Figure 5). Data showed that circulating *C. difficile* isolates from Portugal were genetically diverse, being dispersed along the MST, with some isolates tightly clustering with isolates from Spain (CL01 and CL04). Moreover, we observed that isolates from distinct sources were also dispersed, consistent with a potential association between human and non-human isolates. In fact, when applying a ≤ 2 SNP threshold, seven closely related genetic clusters including isolates from Portugal could be observed (Figure 5, Table 4). Four of these clusters (CL01 to CL04), enrolled isolates from different sources, three of which included isolates from humans and companion animals (CL01, CL03 and CL04). Of note, cluster CL07 was composed by four distinct moxifloxacin resistant isolates collected from canines. Additionally, the largest observed cluster, CL01, enrolled not only isolates collected from different countries and sources, but also isolates spanning a seven-year time period (Table 4).

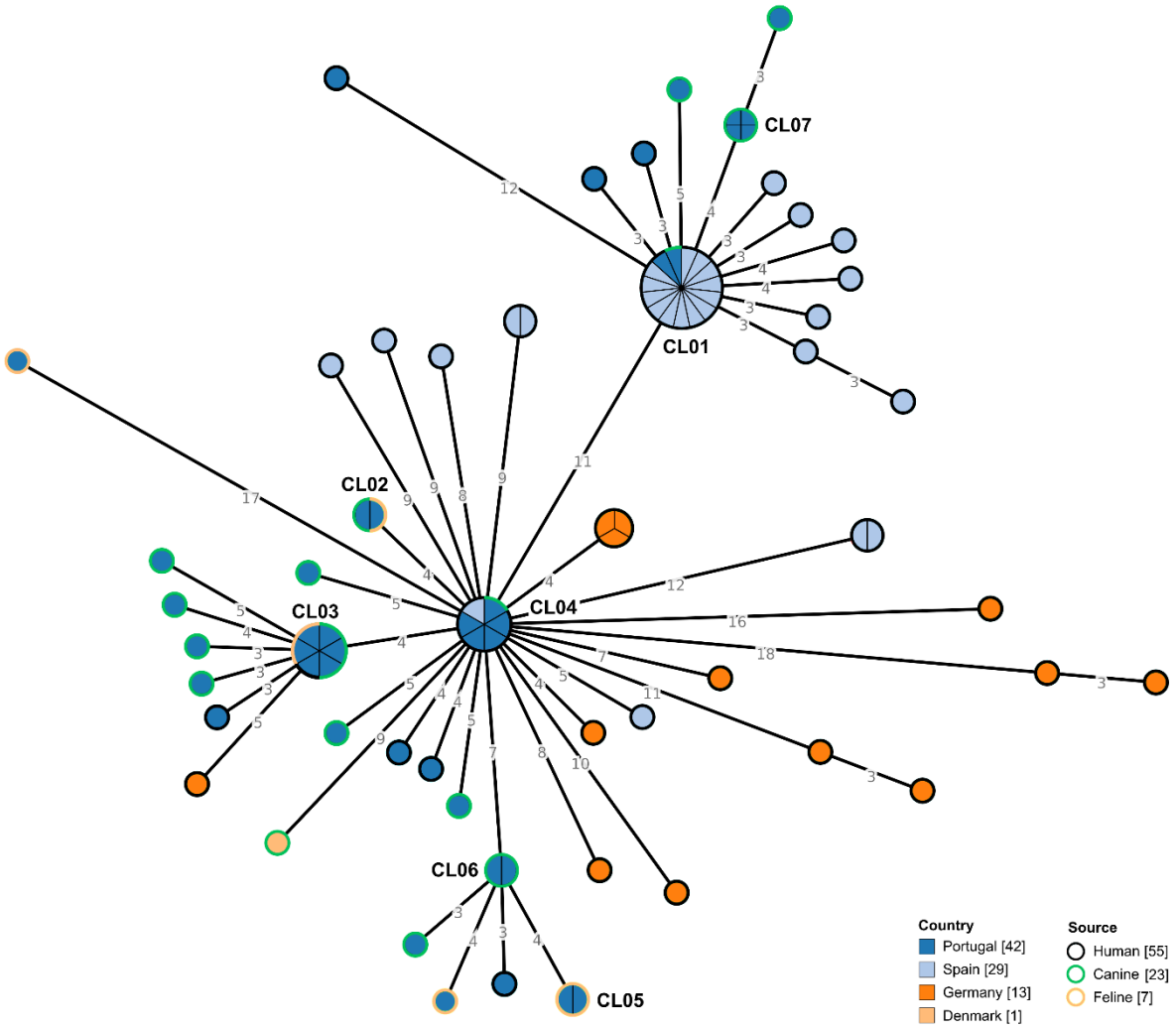


Figure 5: Phylogeny of *Clostridioides difficile* isolates from ribotype 106 used in the present study. The minimum spanning tree (MST) was constructed based on the core-SNP diversity found among 85 isolates, relative to reference genome. All nodes (which represent a unique allelic profile) presenting an SNP distance ≤ 2, representing clusters with potential epidemiological relevance, have been collapsed

for visualization purposes. Nodes are colored according to different countries of origin and their contour colored by respective source. The MST was generated using GrapeTree v1.5.0 software.

Regarding *C. difficile* RT014/020, 41 genomes from isolates collected from distinct sources in Portugal were integrated with 142 genomes previously obtained in several countries (Figure 6). Similarly to what was observed for RT106 isolates, RT014/020 isolates from Portugal presented considerable genetic diversity. Clustering data at a 2 SNP threshold revealed eight distinct genetic clusters enrolling isolates from Portugal, two of which also comprised isolates from other countries (Figure 6 and Table 4), namely Germany, Australia, Italy and Ireland. Three out of the five clusters enrolling isolates from distinct sources linked genomes from companion animals and human infection cases (CL09, CL12, CL14).

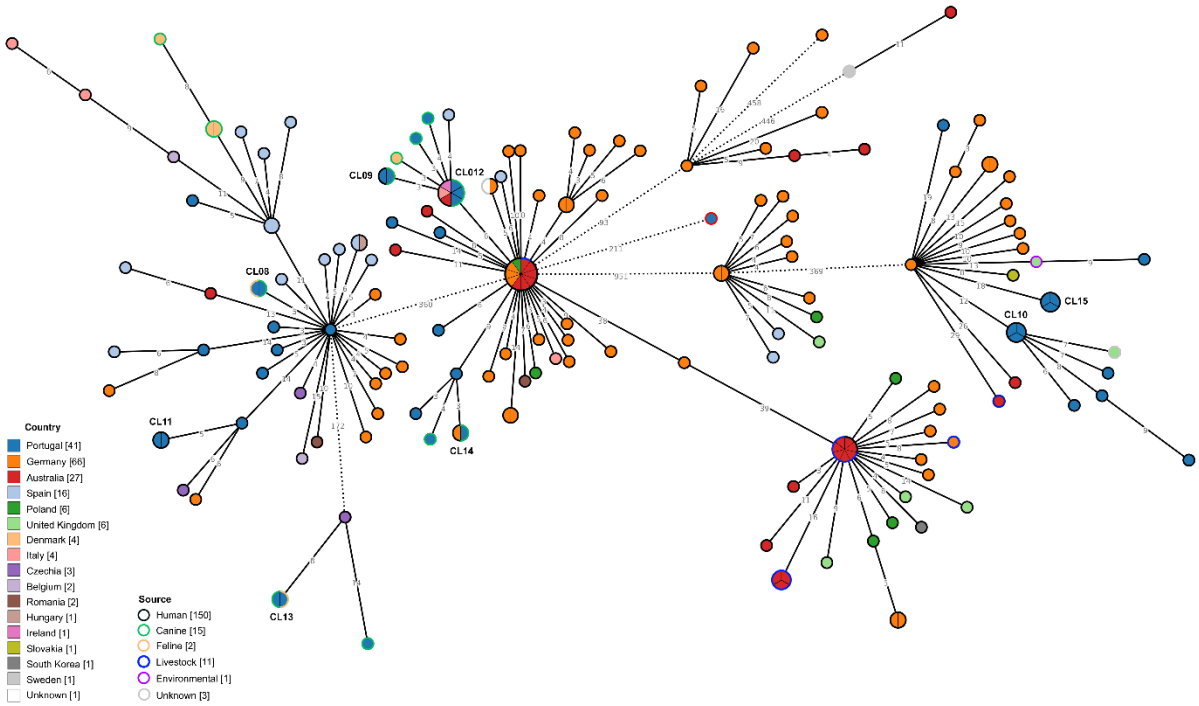


Figure 6: Phylogeny of *Clostridioides difficile* isolates from ribotypes 014/020 used in the present study. The minimum spanning tree (MST) was constructed based on the core-SNP diversity found among 183 isolates, relative to reference genome S-0352 (CP076377.1). All nodes (which represent a unique allelic profile) presenting an SNP distance ≤ 2, representing clusters with potential epidemiological relevance, have been collapsed for visualization purposes. Straight and dotted lines reflect nodes linked with the SNP distances below and above 100 respectively. Nodes are colored according to different countries of origin and their contour colored by respective source. The MST was generated using GrapeTree v1.5.0 software.

Overall, for the 14 genetic clusters including at least two isolates from Portugal, there was overlapping of Portuguese geographical regions, except for three cases (CL03, CL04 and CL09).

Table 4. Genetic clusters including *Clostridioides difficile* isolates from Portugal.

Cluster ID	Cluster length (n° of isolates)	Samples	Ribotype	Country	Source	Collection date	Moxifloxacin Resistance
CL01	15	PT_CD00043, PT_CD00057, ERR3276441, ERR3276506, ERR3278163, ERR3278167, ERR3288184, ERR3288190, ERR3288338, ERR3289201, ERR3289202, ERR3289206, ERR3289207, ERR3289212, ERR3299518	106	Spain (86.7%), Portugal (13.3%)	Human (93.3%), Canine (6.7%)	2015 (46.7%), 2014 (33.3%), 2021 (13.3%), 2016 (6.7%)	No data (86.7%), R (13.3%)
CL02	2	PT_CD00053, PT_CD00055	106	Portugal (100.0%)	Feline (50.0%), Canine (50.0%)	2021 (100.0%)	S (100.0%)
CL03	6	PT_CD00064, PT_CD00030, PT_CD00031, PT_CD00048, PT_CD00049, PT_CD00044	106	Portugal (100.0%)	Canine (50.0%), Feline (33.3%), Human (16.7%)	2021 (66.7%), 2020 (33.3%)	S (83.3%), R (16.7%)
CL04	6	PT_CD00059, PT_CD00060, PT_CD00062, PT_CD00066, PT_CD00046, ERR3288329	106	Portugal (83.3%), Spain (16.7%)	Human (83.3%), Canine (16.7%)	2021 (83.3%), 2014 (16.7%)	S (66.7%), R (16.7%), No data (16.7%)
CL05	2	PT_CD00027, PT_CD00028	106	Portugal (100.0%)	Feline (100.0%)	2020 (100.0%)	S (100.0%)
CL06	2	PT_CD00036, PT_CD00029	106	Portugal (100.0%)	Canine (100.0%)	2021 (50.0%), 2020 (50.0%)	S (100.0%)
CL07	4	PT_CD00050, PT_CD00051, PT_CD00052, PT_CD00054	106	Portugal (100.0%)	Canine (100.0%)	2021 (100.0%)	R (100.0%)
CL08	2	PT_CD00094, PT_CD00095	014/020	Portugal (100.0%)	Feline (50.0%), Canine (50.0%)	2021 (100.0%)	S (100.0%)
CL09	2	PT_CD00086, PT_CD00098	014/020	Portugal (100.0%)	Canine (50.0%), Human (50.0%)	2021 (100.0%)	R (50.0%), S (50.0%)
CL10	3	PT_CD00075, PT_CD00077, PT_CD00085	014/020	Portugal (100.0%)	Human (100.0%)	2021 (100.0%)	S (100.0%)
CL11	2	PT_CD00099, PT_CD00100	014/020	Portugal (100.0%)	Human (100.0%)	2021 (100.0%)	S (100.0%)
CL12	6	PT_CD00089, PT_CD00091, PT_CD00093, ERR1307028, SRR7308801, SRR7309218	014/020	Portugal (50.0%), Australia (16.7%), Ireland (16.7%), Italy (16.7%)	Canine (50.0%), Human (50.0%)	2021 (50.0%), 2013 (33.3%), 2012 (16.7%)	No data (50.0%), S (33.3%), R (16.7%)
CL13	2	PT_CD00108, PT_CD00107	014/020	Portugal (100.0%)	Feline (50.0%), Canine (50.0%)	2021 (100.0%)	S (100.0%)
CL14	2	PT_CD00088, ERR3465438	014/020	Portugal (50.0%), Germany (50.0%)	Canine (50.0%), Human (50.0%)	2021 (50.0%), 2014 (50.0%)	S (50.0%), No data (50.0%)
CL15	3	PT_CD00071, PT_CD00073, PT_CD00074	014/020	Portugal (100.0%)	Human (100.0%)	2021 (100.0%)	S (100.0%)

R – Resistant; S - Susceptible. Genetic clusters were defined at a threshold of ≤ 2 SNPs.

Conclusion

The present study represents an important contribution to the overall knowledge on the epidemiological role of companion animals in CA-CDI and brings awareness to the importance of including companion animals in the One Health research. With the number of animals owning households expected to increase in the coming years it is of paramount importance to clarify their role in community pathogen transmission networks. Further investigation on epidemiologically related animal and human populations is needed to assert a definite transmission route while also assessing efficient and reasonable public health measures to minimize the risks associated with companion animals' waste disposal and advise on hygienic measures towards a safer animal-human interaction.

All the results from this work were published in: *Alves F, Castro R, Pinto M, Nunes A, Pomba C, Moreira O, Silveira L, Gomes JP and Oleastro M (2022). Molecular epidemiology of Clostridioides difficile in companion animals: genetic overlap with human strains and public health concerns. Front. Public Health. doi: 10.3389/fpubh.2022.1070258.*

3. RT 002 (ST8) - Preliminary results of a genetic analysis

Collection

A collection of *C. difficile* RT 002 genomes was created to gain a comprehensive overview of the genetic characteristics of this ribotype lineage. This collection was composed of RT 002 genomes from the partners (SSI, INSA, PI, BfR, FLI; 275 human and 25 non-human *C. difficile* isolates) and genomes belonging to sequence type (ST) 8 from the public database Enterobase. Overall, 540 genomes could be included in the analysis so far. As reference strain, NZ_CP025047 was used.

Characteristics of the genomes

The genome size ranged from 3.8 to 5.7 Mbp. As some of the genomes are quite large, they have to be checked carefully for contaminations in a next step. The genome average nucleotide identity (ANI) showed that all genomes were more than 99.7% identical to the reference strain. A taxonomic classification of the genomes revealed that some genomes had a low percentage for *C. difficile* as first match. This is due to a short contig size of these isolates as short contigs cannot be matched taxonomically. In total, 11,424 different genes were detected (pangenome). 2,645 (23%) of them were assigned to the core genome.

Multi locus sequence typing

Multi locus sequence typing (MLST) revealed that RT 002 isolates belong to ST 8. From 540 genomes, only two had to be assigned to other STs due to differences in one allele each. One isolate assigned to ST 309 showed a different allele in glyA (allele 49 instead of allele 6) and another isolated assigned to ST 611 in recA (allele 47 instead of allele 1).

Analysis of core genome single nucleotide polymorphisms

Depending on the chosen distance threshold, different numbers of clusters were detected. With a threshold of 0 cgSNPs, one cluster could be detected with human and non-human (food) isolates (Figure 7). The human isolates were provided by different partners (SSI and IP), the food isolates were provided by the BfR. If there is an epidemiological connection between these isolates needs further investigation.

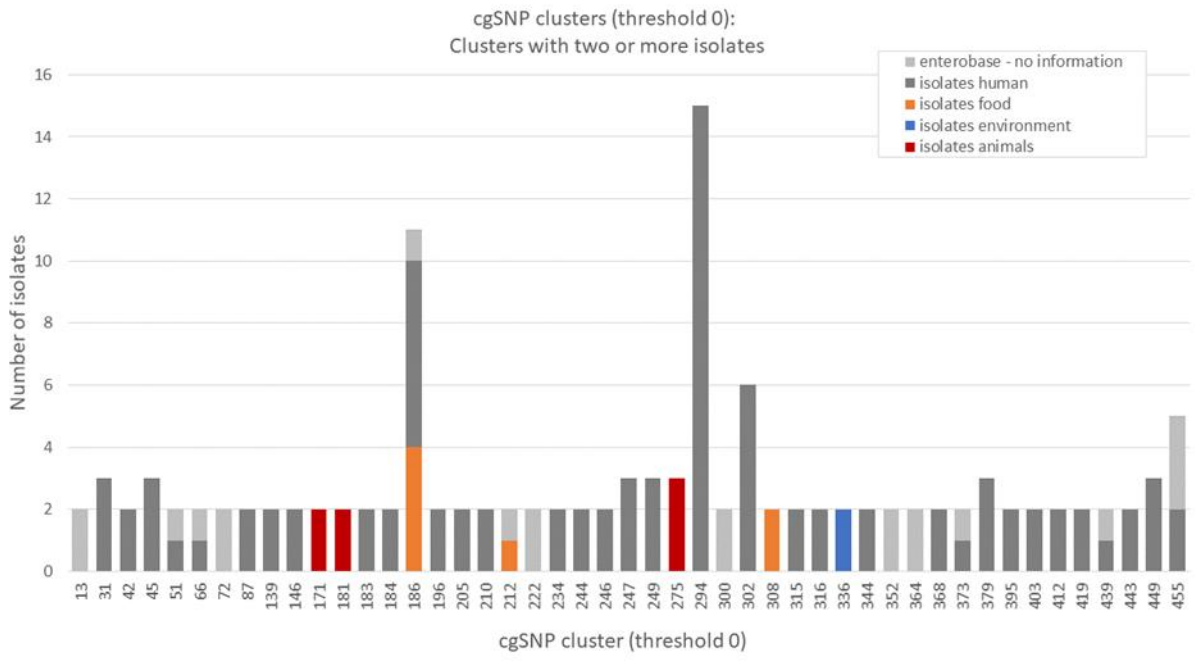
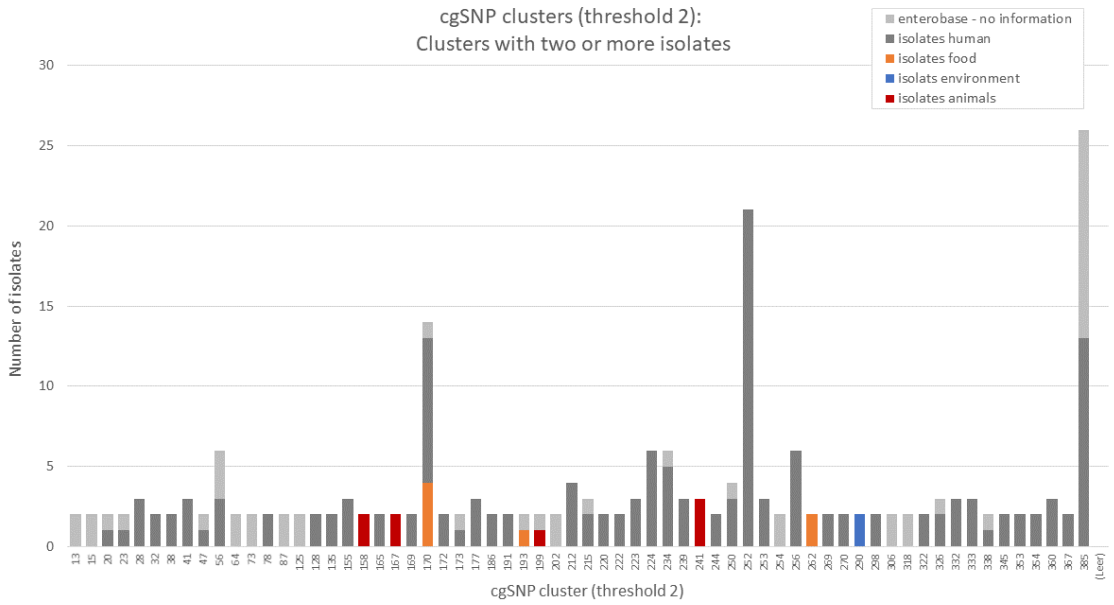


Figure 7

388 clusters could be detected with a threshold of 2 cgSNPs, 22 of these clusters contained three or more isolates (Figure 8). With a threshold of 50 cgSNPs, 529 of the 540 isolates could be assigned to one cluster (six clusters in total). The highest distance between two isolates was 2,002 cgSNP.

Figure 8



Virulence genes

19 different virulence genes were detected. In all isolates, CD0873, CD2831, cap8D, clpP, cwp66, cwp84, fbpA/fbp68, groEL and zmp1 (or parts of them) could be found. CD3246, cbpA and slpA were detected in 539 isolates each, tufa in 538 isolates, iap in 536 isolates, and cwpV in 534 isolates.
The toxin gene toxB was detected in all isolates, toxA in 539 isolates. The binary toxin gene cdtA was detected in one isolate, whereas cdtB was found in all isolates. This is surprising as at least three of the isolates have been negative for both binary toxin genes by PCR. Therefore, the obtained results for virulence genes need to be reviewed carefully.

Antimicrobial resistance

Different antimicrobial resistance determinants could be detected. blaCDD genes and blaR1 could be detected in all isolates. In 99.6% (538/540) of the isolates, blaCDD-1 was present. blaCDD-2 could be found in three isolates. One isolate (ST8-SSI-h155-21) harboured both blaCDD genes (coverage of 40.58% for blaCDD-1, 71.31 % for blaCDD-2) and one isolate (CLO_EA0776AA) possessed two copies of blaCDD-1. Three isolates harboured tet(M). In other three isolates, tet (40) could be found. These isolates possessed lsa, too. The erm(B) gene could be detected in nine isolates, one of them was in possession of tet(M) and mefH, too. The cfr(C) gene was found in one isolate which harboured a tet(M) gene as well. The aminoglycoside resistance gene aadE (named ant(6)-Ia by another database) was detected in nine isolates. Three of them were also positive for the gene aac(6′)-Ie-aph(2″)-Ia. cdeA was detected in all isolates, as well as a vanG gene cluster (vanG, vanRC, vanSC and vanTC). The gene vanZA was found in 91.5% (494/540) of the isolates. abc-f was detected in 98.9% (534/494) of the isolates.

In the gyrase genes, different substitutions leading to amino acid subsitutions in the proteins were detected. In GyrA, the substitution T82I was found in 25 isolates (4.6%). In one isolate, T82V was detected. In GyrB, the substitution D426N was detected in three isolates.
In rpoB, the substitutions R505K (three isolates) and S550Y (one isolate) were detected.
Taking these results together, it seems that blaCDD-1, the vanG gene cluster, vanZA, cdA and abc-f are common in RT 002. Other resistance genes that can be found in C. difficile seem to be rather rare, e.g. tet or erm genes.

Mobile genetic elements
With a self-created database including mobile genetic elements (MGE) described in literature for C. difficile, different transposons, plasmids and phages (or parts of these elements) could be detected. A CTn4-like element was detected in one isolate, CTn6-like elements in three isolates. A Tn5397-like element and a Tn5398-like element were detected in one isolate each. One isolate harboured a Tn6110-like and Tn6111. A Tn6111-like element was detected in another isolate, too. Tn6189-like elements were detected in 102 isolates.
17 different plasmids previously described for C. difficile were detected: pCD630 (8/540), pCD6 (186/540), pCD-ECE2 (3/540), pCD-ECE4 (11/540), pCD-ECE5 (61/540), pCD-ECE6 (55/540), pCDBI1 (473/540), pCD-WTSI1 (164/540), pZJCDC-S82 (163/540), pAK1 (208/540), pAK2 (473/540), LIBA6289 (29/540), unnamed plasmids of strains CDT4 (473/540), CD161 (plasmid 1 (199/540) and 2 (473/540)), FDAARGOS_267 (plasmid 1 (473/540) and 2 (207/540)).
In total, 20 different phages previously described for *C. difficile* were detected. Phage phiCD211 (also named phiCDIF1296T) was found the most frequently (206/540).

Conclusion and outlook
Several virulence genes, antimicrobial resistance determinants, and mobile genetic elements were detected. Whether isolates carrying these elements or element patterns belong to different clusters of RT 002 and whether human and non-human isolates have different characteristics requires further investigation. So far, cgSNP analysis has shown that a clonal cluster includes human isolates and isolates from food. Analysis of cgMLST is still pending, but it is already clear that RT 002 is one of the ribotypes that must be considered important in the context of one health. Further analysis and preparation of a manuscript are in progress.
These results will be part of a manuscript in preparation: *Clostridioides difficile RT002 epidemiology and zoonotic potential. Authors would be: involved FLI staff and WP3 Partners*.

4. RT 049 (ST36) - Preliminary results of a genetic analysis

RT049/ST36 *C. difficile* were the most prevalent strains that were identified in an Estonian agricultural facility (HOAL) over ecosystem boundaries in frame of the WP2/WP3 collaboration, which means that they could be isolated from almost all tested compartments (in animal as well as environmental samples).
So, we aimed at analyzing this particular PCR-ribotype in depth using next-generation sequencing (NGS) and core-genome multilocus sequence typing (cgMLST) to elucidate phylogenetic relationsships.
We therefore collected 153 RT049 strains from the BfR strain collection, FED-AMR sampling campaigns and FED-AMR partner institutes INSA, SSI and FLI as well as from external partners with different origin regarding sample type (animal n=33, human n=109, environment n=10, food n=1) and country (Germany n=6, Estonia n=31, Poland n=2, Denmark n=100, Portugal n=14). RT049 WGS data in Enterobase were not available.
All strains were toxigenic and harbored the toxin genes tcdA and tcdB which are prerequisites to cause human *C. difficile* infections (CDI).
The main sequence type associated with RT049 was ST36 even though we also identified two strains belonging to ST251 (Estonia, Poland) and two strains belonging to ST325 (Portugal).

Genomic sequences were further pairwisely compared using the Ridom™ SeqSphere+ cgMLST scheme based on 2270 core genes. An overview of the resulting minimum spanning tree (MST) is given in Figure 9.

We found four distinct main lineages that differed by more than 50 alleles. Two of these lineages included only strains from Denmark, one lineage with human strains only and the other lineage with both, human and veterinary strains. The other two main lineages contained strains from different countries as well as different ecological background indicating a transmission over ecosystem boundaries.

Furthermore, four clusters with a distance threshold of equal or less than two alleles could be identified containing strains that were highly genetically related. Three small clusters that included two veterinary or human strains each and a further large cluster that included strains with different origin (environment, animal) from the Estonian HOAL.

Antimicrobial resistance genes were determined using the software tool abricate and the AMRFinder database. All RT049 strains were positive for blaCDD, vanG, vanR, vanS, vanT and vanZ1. blaCDD can confer resistence to β-lactam antibiotics while the van-operon in C. difficile is not associated with phenotypic resistance against vancomycin. One human strain from Portugal harbored additional resistance genes: aac(6')-Ie, aph(2'')-Ia, blaCDD, tet(M), tet(W), vanG, vanR, vanS, vanT, vanZ1. While aac(6')-Ie and aph(2'')-Ia can confer resistance against aminoglycosides like gentamycin and kanamycin, tet(M) and tet(W) can be associated with tetracycline resistance. The analysis of associated mobile genetic elements is pending.

In conclusion, we found that RT049 *C. difficile* strains can be devided further by MLST although the vast majority is belonging to ST36. Below the ST-level we were able to distinguish four main lineages. To which extent each lineage has a zoonotic background, is adapted to certain host species / ecological niches or occurs only regionally has to be further investigated. However, the spread of RT049 strains over ecosystem boundaries could be exemplified. A transmission between humans, animals and the environment according to the one-health principle is very likely. Even though antimicrobial resistance does not seem to be a special feature of RT049 strains, we were able to identify an exception that reveals a larger resistance profile most probably by acquisition of mobile genetic elements. Furthermore, all strains contained the classical toxin genes tcdA and tcdB and can therefore represent a biological hazard for human health.

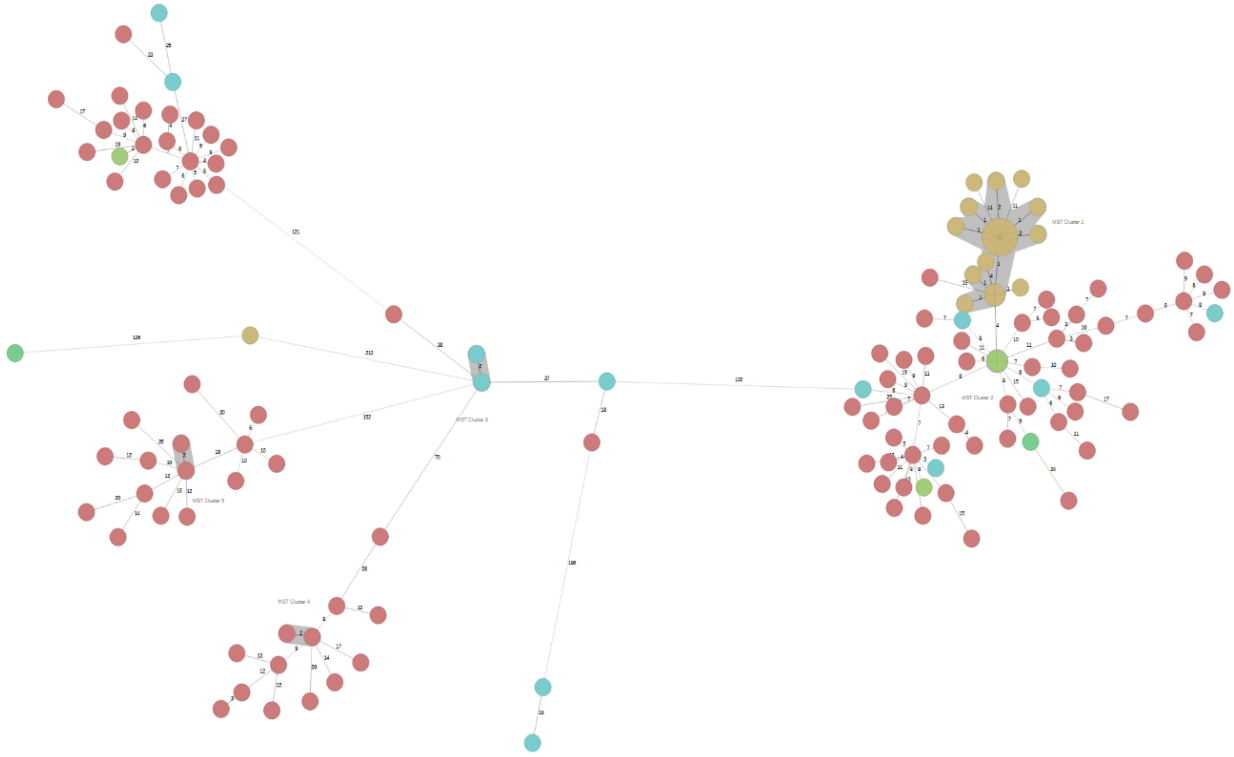


Figure 9: Minimum spanning tree of *Clostridioides difficile* RT049 cgMLST based on 2270 genes. Strains from Denmark are indicated as dots in red, from Estonia in yellow, from Germany in light green, from Poland in dark green and from Portugal in blue. Clusters with a distance threshold of ≤ 2 alleles are highlighted in grey.

These results will be part of a manuscript in preparation: *Maurischat, S., Seyboldt, C., Scholtzek, A., Tenson, T, et al. Transmission and Persistence of Clostridioides difficile within agricultural settings and the environment.*

Concluding remarks

With rising numbers of reported community-acquired infections, research should focus on the role of alternative reservoirs and new transmission networks. Overall, these studies showed genetic overlap between human and non-human *C. difficile* lineages at different One Health settings, supporting the high zoonotic importance of several *C. difficile* lineages, namely from RT078, RT106, RT014/020, RT002.

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