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Special Issue Article

Agricultural fertilization with poultry manure results in persistent environmental contamination with the pathogen *Clostridioides difficile*

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Summary

During a field experiment applying broiler manure for fertilization of agricultural land, we detected viable *Clostridioides* (also known as *Clostridium*) *difficile* in broiler faeces, manure, dust and fertilized soil. A large diversity of toxigenic *C. difficile* isolates was recovered, including PCR ribotypes common from human disease. Genomic relatedness of *C. difficile* isolates from dust and from soil, recovered more than 2 years after fertilization, traced their origins to the specific chicken farm that had delivered the manure. We present evidence of long-term contamination of agricultural soil with manure-derived *C. difficile* and demonstrate the potential for airborne dispersal of *C. difficile* through dust emissions during manure application. *Clostridioides* genome sequences virtually identical to those from manure had been recovered from chicken meat and from human infections in previous studies, suggesting broiler-associated *C. difficile* are capable of zoonotic transmission.

Introduction

The anaerobic gut bacterium *Clostridioides difficile* (also known as *Clostridium difficile* (Lawson *et al*., 2016)) is the most frequent infectious cause of antibiotic-associated diarrhoea and among the leading culprits of healthcare-associated infections (Martin *et al*., 2016). However, modelling studies have suggested that transmission in the community and in the healthcare system were equally relevant for sustaining *C. difficile* in the human population (Durham *et al*., 2016; McLure *et al*., 2019). Patients asymptomatically colonized with *C. difficile* upon hospital admission have a sixfold increased risk of suffering a *C. difficile* infection (CDI) (Zacharioudakis *et al*., 2015), and even without developing CDI themselves they may increase the overall burden of nosocomial CDI significantly by spreading the pathogen to other patients (Longtin *et al*., 2016; Blixt *et al*., 2017; Donskey *et al*., 2018). In addition, CDI occurs independent from healthcare at increasing incidence (Ofori *et al*., 2018), but reservoirs and pathways of transmission outside of the hospital environment are incompletely understood (Warriner *et al*., 2017; Rodriguez Diaz *et al*., 2018).

Toxigenic *C. difficile* seems widespread in various environments, since it was recovered from domestic wastewater (Moradigaravand *et al*., 2018; Numberger *et al*., 2019) and river sediments (Zidaric *et al*., 2010),

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from compost (Janezic *et al*., 2020; Lim *et al*., 2020a), soil (Janezic *et al*., 2016) and root vegetables (Lim *et al*., 2018; Tkalec *et al*., 2019). It was also found to colonize various mammals and birds, including wildlife, pets and livestock (Weese, 2020). Notably, fattening pigs have been proposed as a potential source for transmission of *C. difficile* to humans, since strains with highly related genomes were isolated from both, pigs and farm workers (Knetsch *et al*., 2018). *Clostridioides difficile* was also detected in chicken faeces and chicken meat repeatedly (Zidaric *et al*., 2008; Weese *et al*., 2010; Harvey *et al*., 2011; Abdel-Glil *et al*., 2018; Heise *et al*., 2021), even though there is no evidence for significant CDI in birds (Weese, 2020). Livestock manure often contains *C. difficile* even after being treated by composting or fermentation in biogas plants (Usui *et al*., 2017; Dharmasena and Jiang, 2018; Le Maréchal *et al*., 2020). As a consequence, the disposal of manure or manure-derived products as fertilizer on agricultural land may lead to environmental contamination with *C. difficile* spores. The survival of *C. difficile* in fertilized agricultural soil and its release with surface water runoff or dust has as yet not been investigated, in contrast to other manure-derived pathogens, including *Escherichia coli*, *Salmonella enterica*, *Campylobacter* spp., *Clostridium perfringens* and *Enterococcus faecium* (Blaustein *et al*., 2015; Thiel *et al*., 2020; Siller *et al*., 2021).

The spread of pathogenic bacteria can be tracked by comparing their genome sequences (Croucher *et al*., 2015; Besser *et al*., 2019; Thiel *et al*., 2020). Within the EnteroBase platform, we have recently established a publicly accessible database for *Clostridioides* genomic data that currently (May 2021) contains 22 016 draft genomes and their associated metadata (Frentrup *et al*., 2020). Standardized sequence data assembly and quality control in conjunction with core-genome multilocus sequence typing (cgMLST) and hierarchical clustering of cgMLST allelic profiles - as implemented in EnteroBase - facilitates the detection of *C. difficile* spread (Frentrup *et al*., 2020). Hierarchical clusters (HC) are chains of genomes with specified pairwise distances, which represent populations of *C. difficile* at various epidemiological levels, from transmission chains to epidemics to endemic occurrence (Table 1). For example, we have recently demonstrated that isolates from transmission chains frequently can be identified by being related at the HC2 level, i.e. they constitute chains of genomes with pairwise differences of maximally two cgMLST alleles (Frentrup *et al*., 2020). Moreover, widespread epidemic strains, e.g. the fluoroquinolone-resistant clones of PCR ribotype 027, commonly are related at the HC10 level. In contrast, PCR ribotypes (RTs) represent widely spread endemic strains that correlate well with clusters at the HC150 level (which we dubbed 'core-genome sequence typing complexes'; CC) (Frentrup *et al*., 2020).

Table 1. Hierarchical clusters of *C. difficile* based on genomic distances (Frentrup *et al*., 2020).

HC level	Epidemiological correlate
HC2	Transmission, outbreak
HC10	International epidemic
HC150 ('CC')	Endemic occurrence, PCR ribotype

In the present study, we detected the persistence of viable *C. difficile* in agricultural soil for several years following its fertilization with manure from broiler chickens. Genomic relatedness of *C. difficile* isolates from soil and from dust released during the fertilization process traced their origins to the specific chicken farm that had delivered the manure.

Results

We isolated a total of 278 *C. difficile* isolates and sequenced their genomes (Suppl. Table S1). Of these isolates, 146 had been recovered from chicken manure and 132 were from dust and from manure-fertilized soil respectively.

Diversity of C. difficile *isolates in chicken manure*

Chicken manure was sampled at three different locations, including two farms and a manure trading cooperative. Altogether 146 *C. difficile* isolates were obtained from manure samples (Table 2) and their genomes were sequenced. Genomic data indicated that 98% of the isolates carried both toxin genes, *tcdA* and *tcdB* (Fig. 1A), and only three isolates were non-toxigenic. Analysis of genome sequences with EnteroBase showed that manure isolates were related to 13 CCs (i.e. HC at the level HC150), which we had previously shown to correlate well with PCR ribotypes (Fig. 1A) (Frentrup *et al*., 2020). The majority of isolates (94%) from Farm 1 were related to CC3 (Table 2; Fig. 1A), which corresponds to PCR ribotype 001 (Frentrup *et al*., 2020), and repeated samplings showed that this predominance of CC3 at Farm 1 was evident over a period of at least 1 year (Fig. 1B). In contrast, only one isolate (4%) from Farm 2 was CC3, and none from the manure trader (Fig. 1A). Instead, isolates from the latter two suppliers were distributed among a number of different CCs, the most predominant of which were CC71 (RT014/020), CC88 (RT014), CC2 (RT002), CC86 (RT005) and CC391 (RT081) (Fig. 1A).

Close genomic relationships identify source of environmental C. difficile

A 2.1-ha agricultural field was fertilized with 12 tons of poultry manure from Farm 1 (on 31 May 2017) (Thiel *et al*., 2020). Prior to fertilization, our enrichment

Table 2. Core-genome sequence type complexes (CC)

CC	PCR ribotype	Farm 1	Farm 2	Trader
CC2	RT002	4 (4.3%)	1 (4.2%)	7 (25%)
CC3	RT001	88 (93.5%)	1 (4.2%)	0
CC71	RT014/020	0	10 (41.7%)	3 (10.6%)
CC86	RT005	1 (1.1%)	2 (8.3%)	4 (14.3%)
CC88	RT014	0	2 (8.3%)	10 (35.7%)
CC391	RT081	0	5 (20.8%)	0
CC5408	RT029	0	1 (4.2%)	0
CC5410	novel	0	2 (8.3%)	0
CC207	RT003	1 (1.1%)	0	0
CC34	RT014	0	0	1 (3.6%)
CC596	RT011/049	0	0	1 (3.6%)
CC645	RT029	0	0	1 (3.6%)
CC1643	RT011/049	0	0	1 (3.6%)
Total		94 (100%)	24 (100%)	28 (100%)

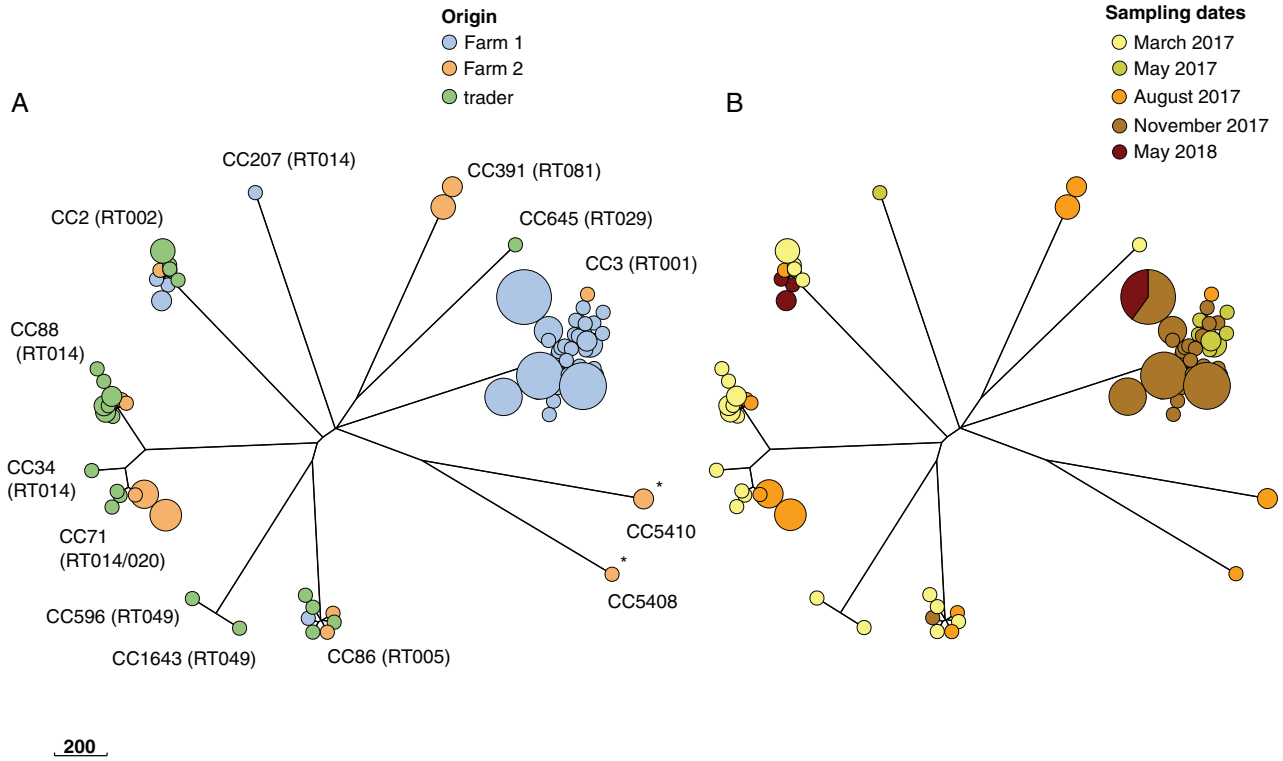

Fig. 1. Rapid-neighbour-joining phylogenetic tree based on cgMLST allelic differences between *C. difficile* isolates ($n = 146$) from manure samples. Node sizes indicate the number of included entries and the scale bar indicates the branch length corresponding to sequence differences at 200 cgMLST loci.
A. Colours indicate the origins of manure. All genomes carried intact *tcdA* and *tcdB* toxin genes, except those marked with asterisks. CC, core-genome sequence-typing complex; RT, PCR ribotype.
B. Same tree as in Fig. 1A, with colours indicating sampling dates.

approach had failed to detect any *C. difficile* in soil from this field. After fertilization, however, *C. difficile* could be detected consistently in soil samples collected at multiple points in time for up to 143 weeks (Fig. 2). Moreover, one dust sample collected during manure spread by using an aerosol collection device (Thiel *et al*., 2020) at the edge of the field tested positive for *C. difficile* by enrichment (Fig. 2). Of note, *C. difficile* was detected in soil and dust by enrichment culture only, whereas cultivation and quantification by direct plating on selective agar medium were not successful. Altogether, we collected 129 *C. difficile* isolates from fertilized soil and from dust, three from poultry faeces from Farm 1, and 12 from manure from Farm 1 (on 30 May 2017). Bacterial genome

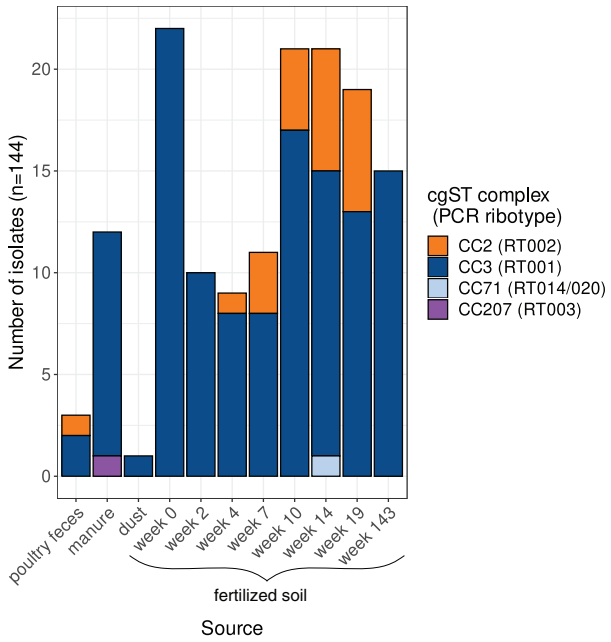

Fig. 2. Distribution of isolates recovered from chicken faeces and manure from Farm 1 and from samples collected during the field experiment ($n = 144$). Colours represent CCs, which were determined based on cgMLST allelic profiles in EnteroBase.

sequencing and cgMLST-based hierarchical clustering analysis with EnteroBase (Frentrup *et al*., 2020) resulted in three clusters of closely related *C*. *difficile* isolates (HC2_1232, HC2_5435, HC2_5465; Fig. 3) and four singletons. SNP analysis based on mapping the sequencing reads to a reference genome confirmed this result by demonstrating that study isolates within those three HC2 clusters differed from each other by maximally two genome-wide SNPs, with the exception of a single isolate from soil (Suppl. Fig. S1). Hierarchical clustering at the level HC2 was previously shown to indicate close genomic relationships of *C*. *difficile* isolates, for example correlating with events of transmission between hospital patients (Frentrup *et al*., 2020).

Two clusters (HC2_1232, HC2_5435) indicated close relationships between genomes from two or more different sources, including chicken faeces collected at Farm 1, manure from Farm 1, dust collected during the application of manure to the field and fertilized soil from multiple points in time (Table 3; Fig. 3). This result confirmed that the *C*. *difficile* strains that were recovered during and after fertilization indeed originated from Farm 1, i.e. they had been disseminated onto the agricultural field through the fertilization process. These close genomic relationships were found among *C*. *difficile* isolates from all soil samples, indicating the persistence of viable, manure-derived *C*. *difficile* in the soil for up to 143 weeks after fertilization (Fig. 3). Likewise, the detection of closely related *C*. *difficile* in mineral dust showed that viable cells of the pathogen got aerosolized during the fertilization process and transported in an ascending dust plume at a distance of at least 20 m from the applying tractor (Fig. 3).

PCR ribotypes and antibiotic susceptibilities

Clostridioides difficile isolates ($n = 19$) selected to represent sources (i.e. manure from the different suppliers, fertilized soil, and dust) and genomic diversity (at the level of CCs) proved to be phenotypically susceptible to the antibiotics vancomycin, metronidazole, moxifloxacin, clindamycin and tetracycline (Table 4). By scanning the genome assemblies from all 278 *C*. *difficile* isolates in this study, we did not find any antibiotic resistance genes or mutations known to confer antibiotic resistance (Alcock *et al*., 2020). In addition, our specific analysis indicated that none of the genome sequences carried resistance-causing mutations in the gyrase gene *gyrA* (Zaiß *et al*., 2010), confirming the lack of fluoroquinolone resistance in our strain collective (not shown). PCR ribotypes determined in the laboratory were fully concordant with ribotype predictions based on hierarchical clustering in EnteroBase (Table 4).

Closely related clinical and poultry meat isolates

Hierarchical clustering of cgMLST allelic profiles in EnteroBase routinely determines genomic relationships at multiple phylogenetic levels among all >20 000 entries in the *Clostridioides* database (Frentrup *et al*., 2020). Remarkably, a limited number of genome sequences from several previous studies were closely related (at HC2 level) to those from Farm 1 (Fig. 3 and Suppl. Table S2). These include *C*. *difficile* genome sequences recovered from retail chicken meat ($n = 7$; Fig. 3), which had been purchased in one region in Germany (Berlin and Brandenburg) but had been produced in a number of different cutting plants in Germany and the Netherlands (Heise *et al*., 2021). Additional closely related genomes originated from isolates from human patients suffering from CDI in Germany ($n = 2$), the Netherlands ($n = 5$) and Hungary ($n = 1$; Fig. 3). Of note, these genomic similarities were not due to impaired quality of the sequence data, since >99% of cgMLST alleles were successfully called for all genome sequences. Moreover, no genes of the whole-genome MLST set (Frentrup *et al*., 2020) were differentially present (not shown), indicating that accessory genomes were virtually identical among all these isolates, too. SNP analysis equally indicated close relatedness (≤2 genome-wide SNPs) between 13 (87%) of those database genomes and genomes from Farm 1 isolates. All these genome sequences were related to CC3 (Fig. 3), which is associated predominantly with PCR ribotype 001 (Frentrup *et al*., 2020).

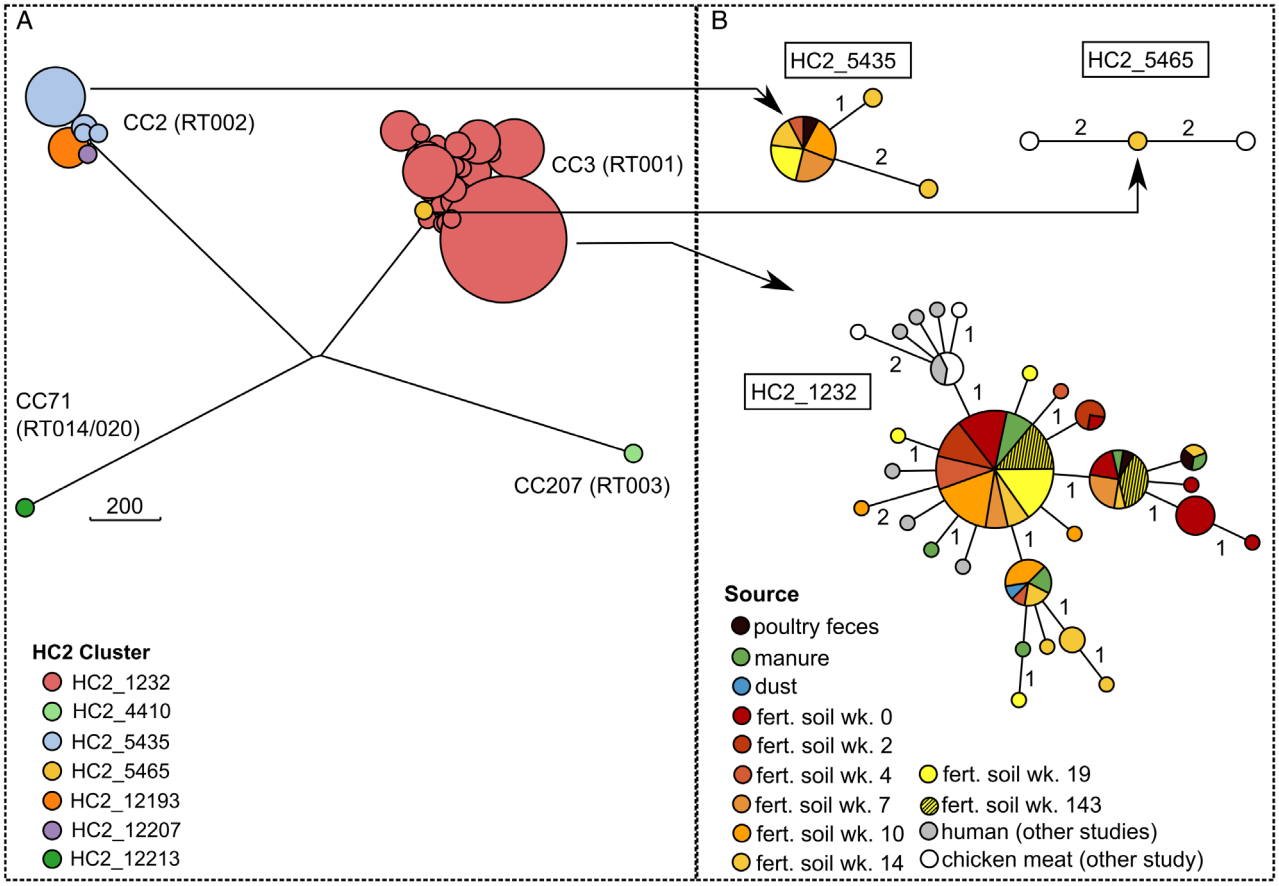

Fig. 3. A. Rapid-neighbour-joining phylogenetic tree based on cgMLST allelic profiles from all isolates ($n = 144$) sampled during the field experiment. Colours indicate HC2 clusters and node sizes indicate the number of included entries. The scale bar indicates the branch length corresponding to sequence differences at 200 cgMLST loci.
B. Minimum-spanning trees for three HC2 clusters. Numbers on branches indicate the number of cgMLST allelic differences and colours represent the source of isolates.

Table 3. The number of *C. difficile* isolates in each HC2 cluster.

Source	HC2_1232	HC2_4410	HC2_5435	HC2_5465	HC2_12193	HC2_12207	HC2_12213
Poultry faeces	2	0	1	0	0	0	0
Manure	11	1	0	0	0	0	0
Dust	1	0	0	0	0	0	0
Fert. soil wk. 0	22	0	0	0	0	0	0
Fert. soil wk. 2	10	0	0	0	0	0	0
Fert. soil wk. 4	8	0	1	0	0	0	0
Fert. soil wk. 7	8	0	3	0	0	0	0
Fert. soil wk. 10	17	0	3	0	1	0	0
Fert. soil wk. 14	13	0	4	1	2	0	1
Fert. soil wk. 19	13	0	3	0	2	1	0
Fert. soil wk. 143	15	0	0	0	0	0	0

Discussion

Chicken manure carried diverse C. difficile*, including clinically relevant strains*

Almost all *C. difficile* isolates from manure in our study carried the *tcdA* and *tcdB* genes in their genomes, and hence must be considered fully virulent and able to cause gastrointestinal disease in humans. This result is in concordance with most previous studies on poultry-associated *C. difficile* (e.g. Dharmasena and Jiang, 2018; Berger *et al*., 2020; Le Maréchal *et al*., 2020; Heise *et al*., 2021) even though there is little evidence

Table 4. Genotypes and antibiotic susceptibilities of 19 selected *C. difficile* isolates.

Isolate	Source	Origin	CC (RT predicted[c]|PCR)	MIC[a] (μg ml^{-1})					Gene content (predicted[b]|PCR)			
				VAN	MTZ	MXF	CLI	TET	*tcdA*	*tcdB*	*cdtA*	*cdtB*
CD-17-00892	Manure	Trader	CC88 (RT014|RT014)	0.5	0.094	0.75	1.5	0.016	+	+	–	–
CD-17-01035	Manure	Trader	CC1643 (RT011/049|RT049)	0.5	0.125	0.75	3	0.047	+	+	–	–
CD-17-01037	Manure	Trader	CC34 (RT014|RT014)	0.5	0.125	0.75	2	0.032	+	+	–	–
CD-17-01039	Manure	Trader	CC645 (RT029|RT029)	0.75	0.125	0.75	0.5	0.047	+	+	–	–
CD-17-01040	Manure	Trader	CC596 (RT011/049|RT049)	0.5	0.125	0.75	3	0.047	+	+	–	–
CD-17-01068	Manure	Farm 1	CC207 (RT003|RT003)	0.38	0.094	1	0.5	0.023	+	+	–	–
CD-17-01070	Dust	Field experiment	CC3 (RT001|RT001)	0.5	0.094	0.5	1.5	0.032	+	+	–	–
CD-17-01381	Manure	Farm 2	CC71 (RT014/020|RT014)	0.75	0.125	0.75	1.5	0.75	+	+	–	–
CD-17-01390	Manure	Farm 2	CC391 (RT081|RT081)	0.5	0.125	0.75	2	0.032	+	+	–	–
CD-17-01395	Manure	Farm 2	CC5408 (n.a.|RT029)	0.75	0.125	1	3	0.032	–	–	–	–
CD-17-01424	Manure	Farm 2	CC5410 (n.a.|novel)	0.38	0.032	1	2	0.023	–	–	–	–
CD-17-01524	Manure	Farm 1	CC86 (RT005|RT005)	1	0.19	0.75	6	0.032	+	+	–	–
CD-18-00685	Manure	Farm 1	CC2 (RT002|RT002)	0.5	0.064	1	2	0.023	+	+	–	–
CD-19-00355	Fert. soil wk. 7	Field experiment	CC2 (RT002|RT002)	0.5	0.125	0.75	2	0.064	+	+	–	–
CD-19-00409	Fert. soil wk. 14	Field experiment	CC71 (RT014/020|RT014)	0.75	0.125	1	4	0.032	+	+	–	–
CD-19-00417	Fert. soil wk. 14	Field experiment	CC3 (RT001|RT001)	0.5	0.19	0.5	1.5	0.047	+	+	–	–
CD-19-00426	Fert. soil wk. 19	field experiment	CC2 (RT002|RT002)	0.38	0.25	1	4	0.047	+	+	–	–
CD-19-00513	Manure	Farm 1	CC3 (RT001|RT001)	0.75	0.75	0.5	4	0.047	+	+	–	–
CD-20-00542	Fert. soil wk. 143	Field experiment	CC3 (RT001|RT001)	0.75	0.75	0.75	6	0.047	+	+	–	–

[a]VAN: vancomycin; MTZ: metronidazole; MXF: moxifloxacin; CLI: clindamycin; TET: tetracycline; *tcdA*: gene encoding for toxin A; *tcdB*: gene encoding for toxin B; *cdtA* and *cdtB*: genes encoding for the binary toxin.
[b]Toxin gene prediction was based on corresponding wgMLST loci (+=present; –=absent).
[c]Ribotypes were predicted based on hierarchical clustering in EnteroBase.

that *C. difficile* may cause disease in birds (Weese, 2020).

In manure samples from three suppliers, we found a total of 13 CCs (core-genome sequence-type complexes) of *C. difficile*. CCs correlate well with PCR ribotypes (Frentrup *et al*., 2020) (Table 4), and RTs 001, 014/020 and 005 have been reported from poultry faeces (Indra *et al*., 2009; Hussain *et al*., 2016; Abdel-Glil *et al*., 2018; Le Maréchal *et al*., 2020) and from broiler meat (De Boer *et al*., 2011; Tkalec *et al*., 2020) in the past. In our manure samples, the most predominant strains were CC3 (RT001), CC71 (RT014/020) and CC88 (RT014). Remarkably, these are also among the most prevalent strains causing human CDIs in Europe (Davies *et al*., 2016). However, our isolates from broiler chickens were not resistant to fluoroquinolones or clindamycin, in contrast to the vast majority of clinical RT001 isolates from human CDI (Zaiß *et al*., 2010; Eyre *et al*., 2018). This striking difference in antibiotic resistances suggests that *C. difficile* RT001 in chickens constitutes a population separate from the epidemic RT001 strain causing healthcare-associated CDI in humans, with limited exchange. This notion was confirmed by hierarchical clustering of genome sequences, which indicated that all our CC3 *C. difficile* from broiler manure ($n = 199$) were related to a single HC10 cluster (HC10_783; Suppl. Table S1) that currently includes only 15 (7%) human-associated *C. difficile* isolates in EnteroBase. Such a separation was not observed for RT014/020, which is antibiotic resistant more rarely (Zaiß *et al*., 2010; Eyre *et al*., 2018), and where 13 isolates from broilers were affiliated to nine different HC10 clusters (Suppl. Table S1), the larger of which included numerous isolates from diverse host species and geographic origins. Fluoroquinolone and clindamycin resistance in poultry-associated *C. difficile* has occasionally been reported (from the USA and Zimbabwe (Harvey *et al*., 2011; Dharmasena and Jiang, 2018; Berger *et al*., 2020)). Since macrolides and fluoroquinolones are the two antibiotics most heavily used in the poultry industry in Europe, and resistance against these drugs is widespread among other gastrointestinal pathogens from chickens (Roth *et al*., 2019), lowered susceptibilities might also have been expected from broiler-associated *C. difficile*, but yet this was not detected in our samples. Hence, while the widespread use of antibiotics is driving the increasing spread of *C. difficile* in both humans and livestock (He *et al*., 2013; Spigaglia *et al*., 2018; Dingle *et al*., 2019), we found no evidence of such dynamics in poultry to date.

Long-term persistence of manure-derived C. difficile *in fertilized agricultural soil*

Clostridioides difficile has been reported from a wide range of different environmental samples, including soil (Rodriguez Diaz *et al*., 2018). To our best knowledge, however, our study is the first to use genome sequence analysis to trace environmental *C. difficile* back to its source. As one result, we show that *C. difficile* in fertilized soil indeed originated from chickens in Farm 1. The field was not fertilized or agriculturally used between sampling time points. Hence, our field experiment demonstrated that manure-derived *C. difficile* remained viable in fertilized soil over the entire study period, i.e. for at least 143 weeks, or almost 3 years. The continued bacteriological detection of *C. difficile* in all samples investigated suggested that its survival may be much longer than the sampling period, even though precise extrapolation was not possible due to the failure of quantitative cultivation. The observed long-term contamination of the soil certainly was enabled by the ability of *C. difficile* to produce endospores, which can stay viable for many years (Yang and Ponce, 2011). In contrast, it is not known if these bacteria are able to perform much metabolic activity or even proliferate under ambient conditions in the soil, but their known physiology is adapted to life in the intestines of warm-blooded animals.

We previously reported that chicken manure carried additional pathogens, including *Enterococcus faecium* and extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* (Thiel *et al*., 2020). However, ESBL *E. coli* died off within a few days during manure storage (Siller *et al*., 2020) and enterococci rapidly declined in soil within weeks after fertilization (Thiel *et al*., 2020). In the present study, in contrast, we demonstrate that viable *C. difficile* remained detectable in fertilized soil for several years and hence represented a long-lasting contamination.

Potential for long-distance dispersal of C. difficile

Hierarchical clustering indicated that altogether 15 entries in the EnteroBase *Clostridioides* database shared identical HC2 clusters (HC2_1232, HC2_5465) with isolates from Farm 1, i.e. they had highly similar cgMLST profiles with at most two allelic differences, despite their origins from unrelated, previous studies. Seven of these isolates had been recovered from retail chicken meat from various cutting plants in Germany and the Netherlands (Heise *et al*., 2021), indicating widespread dissemination of *C. difficile* by the poultry industry. Furthermore, the occurrence of the same closely related clone in human CDI in Germany, Hungary and the Netherlands indicates that this strain is able to cause human disease (HC2_1232, Fig. 3). Consequently, this *C. difficile* HC2 clone poses a risk of zoonotic transmission.

It should be noted that pathogen genomic similarity alone does not prove direct transmission between remote places, but should be interpreted with particular care in the absence of additional, epidemiological evidence

(Besser *et al*., 2019). However, several plausible scenarios for long-distance transport of poultry-associated *C*. *difficile* exist. Chicken meat contaminated with *C*. *difficile* (De Boer *et al*., 2011; Harvey *et al*., 2011; Candel-Pérez *et al*., 2020; Heise *et al*., 2021) gets distributed to customers through widely ramified retail chains. Similarly, pork products (i.e. meat or manure) were suspected to promote the long-distance spread of *C*. *difficile*, after closely related *C*. *difficile* genomes had been detected in fattening pigs and humans across large geographic distances, without any documented epidemiological connections (Knetsch *et al*., 2018; Knight *et al*., 2019). Another potential path for the long-range dissemination of livestock-associated *C*. *difficile* may be the transport of colonized, live animals, e.g. from farms to slaughterhouses (Heise *et al*., 2021). Potentially even more important is the globalized structure of the poultry industry, which ships industrially produced broiler chicks by airfreight for stocking fattening farms globally (Lowder *et al*., 2009). It would be interesting to investigate the colonization status of chickens upon their arrival at fattening farms.

In addition, here we show that mineral dust from agricultural operations may carry aerosolized, manure-derived *C*. *difficile*. This dust may stay airborne for several days and during this time may get transported over several hundred kilometres, depending on atmospheric conditions (Thiel *et al*., 2020; Faust *et al*., 2021). Poultry manure is particularly prone to aerosolization due to its high dry-matter content (Kabelitz et al., 2020; Thiel *et al*., 2020; Kabelitz *et al*., 2021) and therefore, its application for fertilization of agricultural fields likely contributes to the airborne dispersal of chicken-associated *C*. *difficile* over long distances. Aerosolized *C*. *difficile* is considered a potential source of human infection when inhaled (Best *et al*., 2010), similar to other enteric pathogens (Jahne *et al*., 2015). Hence, *C*. *difficile* in agricultural dust may represent a risk of airborne zoonotic transmission. Taken together, our results corroborate the relevance of a 'One Health' approach for curbing the spread of *C*. *difficile* between human, livestock and environmental reservoirs (Lim *et al*., 2020b).

Experimental procedures

Manure samples

To capture the diversity of *C*. *difficile* isolates in manure samples, five samplings were performed on three different sites. Manure samples from two broiler fattening farms and one manure trading cooperative were investigated. In addition, chicken faeces were sampled by collecting 30 chicken droppings from each of 11 stables in Farm 1. Farm 1 is an intensive poultry-fattening farm in Brandenburg, Germany, housing about 19 000 animals per stable on wood pellets. Manure from this farm was sampled three times (30 May 2017, 8 November 2017 and 19 May 2018). In Farm 2, which is located in Saxony-Anhalt, Germany, manure was collected in four different stables on 14 August 2017. Manure from the trader was sampled on 27 March 2017.

Field experiment

In a field experiment, 12 tons of chicken manure from Farm 1 (sampled on 30 May, 2017, see above) were applied to a 2.1-ha agricultural field, which had not been fertilized with animal manure for 15 years. Details of this experiment have been published previously (Thiel *et al*., 2020). Briefly, dust particles that were released during the fertilization process were collected by impingement into 5 ml phosphate-buffered saline (PBS) at a height above ground of 1.50 m and at a distance from the tractor of 20, 50 and 100 m respectively. Soil samples were taken on three representative sites on the field site prior to fertilization, directly after, and 2, 4, 7, 10, 14, 19 and 143 weeks later. Each sample consisted of a mixture of five shovels of soil that were taken from the upper 5 cm around the same spot. The samples were stored at 4°C and analysed within 24 h (Thiel *et al*., 2020).

Isolation of C. difficile *isolates*

Ten grams of poultry faeces, manure and soil samples were mixed with 90 g Luria–Bertani broth (Roth) each and subsequently homogenized for 30 s with a bag mixer (Interscience). After sedimentation of coarse particles (30 min, room temperature), supernatants and impingement suspensions from the aerosol collector were diluted to extinction with PBS and subsequently streaked on ChromID *C*. *difficile* agar (Biomérieux). After incubation at 37°C for 24 h, *C*. *difficile* colonies were identified by species-specific PCR (locus TR10) (Zaiß *et al*., 2009). Of note, this direct plating approach successfully yielded *C*. *difficile* cultures only from faeces and manure samples, whereas enrichments were required for cultivation from soil (Suppl. Table S1). For enrichment cultures, 0.5 ml of suspensions were added to 10 ml brain heart infusion (BHI) broth (Roth) supplemented with 0.1% taurocholic acid (Sigma), 0.1% cysteine (Sigma) and *C*. *difficile* selective Supplement (Oxoid) in Hungate tubes (Janezic *et al*., 2018). After 7 days of incubation at 37°C, an ethanol shock was performed by adding an equal amount of absolute ethanol to 0.5 ml culture and incubation for 1 h at room temperature. The culture was centrifuged at 2500*g* for 5 min, the resulting cell pellet was resuspended in 200 µl PBS, and 100 µl were plated on

ChromID *C. difficile* agar and incubated at 37°C for 24 h. Again, bacterial colonies were tested by *C. difficile*-specific PCR (Zaiß *et al*., 2009).

Antibiotic susceptibility testing

Isolates from agar plates were transferred to anaerobic BHI broth (Roth) in Hungate tubes and grown for 2 days at 37°C. Subsequently, the culture was diluted 1:5 with PBS and 100 µl was spread on Columbia blood agar (Oxoid). For each antimicrobial agent, an E-test strip was applied to the agar surface, followed by 24 h of incubation at 37°C. The tests were interpreted visually by reading the minimum inhibitory concentration (MIC). MICs were determined for vancomycin, metronidazole, moxifloxacin (Biomérieux), clindamycin and tetracycline (Liofilchem). For interpretation, MIC breakpoints for antibiotic resistance were applied according to Pirš *et al*. (2013): metronidazole, ≥2 µg ml^{-1}; vancomycin, ≥2 µg ml^{-1}; moxifloxacin, ≥4 µg ml^{-1}; clindamycin, ≥8 µg ml^{-1}; tetracycline, ≥16 µg ml^{-1}.

PCR ribotyping

PCR ribotyping of 19 selected *C. difficile* isolates was performed as reported previously (Indra *et al*., 2008), applying capillary electrophoresis and the Webribo database (https://webribo.ages.at/).

Whole-genome sequence analyses

Genomic DNA was extracted by using the DNeasy Blood & Tissue kit (Qiagen), libraries were prepared as described previously (Steglich *et al*., 2018) and sequenced on an Illumina NextSeq 500 machine using a Mid-Output kit (Illumina) with 300 cycles. Illumina sequencing reads were uploaded to EnteroBase (http://enterobase.warwick.ac.uk/) and assembled with the embedded standardized pipeline (Frentrup *et al*., 2020). Thirty-two sequences did not pass the quality check in EnteroBase (Frentrup *et al*., 2020) and were excluded from further analyses. For 278 genomes, cgMLST allelic profiles (>99% complete) were determined and cgMLST-based hierarchical clustering performed using EnteroBase tools. To visualize genomic relatedness, rapid-neighbour-joining and minimum-spanning trees were calculated applying GrapeTree (Zhou *et al*., 2018; Frentrup *et al*., 2020). PCR ribotypes were predicted based on genomic relatedness at the level HC150 (i.e. HC of genome sequences with pairwise differences of maximally 150 cgMLST alleles; for details see Frentrup *et al*., 2020).

Genomic relatedness was also determined based on SNP analysis. To this end, sequencing reads were mapped to the genome sequence from strain R20291 (accession number FN545816) by using BWA-MEM (v0.7.12) and sequence variation was detected by using VarScan2 (v2.3) as described previously (Steglich *et al*., 2018). Sequence variation resulting from homologous recombination was detected by using ClonalFrameML and removed prior to determination of pairwise sequence differences (Didelot and Wilson, 2015).

Genome assemblies from all 278 *C. difficile* isolates were scanned for genes and mutations known to confer antibiotic resistance by using the Resistance Gene Identifier software and the Comprehensive Antibiotic Resistance Database (CARD) (Alcock *et al*., 2020). In addition, sequences of the *gyrA* gene (cgMLST locus CD630_00060) were scanned for the mutations Thr-82-Ile and Asp-71-Glu, which are associated with fluoroquinolone resistance in *C. difficile* (Zaiß *et al*., 2010).

All genome sequencing data were submitted to the European Nucleotide Archive (ww.ebi.ac.uk/ena) under the study accession number PRJEB42049. A list of all analysed genomes can be found in Supplementary Table S1.

Detection of toxin genes

DNA from selected isolates ($n = 19$) was tested for the presence of toxin genes *tcdA*, *tcdB*, *cdtA* and *cdtB* by PCR (Persson *et al*., 2008). The presence or absence of toxin genes *tcdA* and *tcdB* was determined for all genomes in this study ($n = 278$) based on allelic numbers for toxin gene loci in EnteroBase. As for any other protein-coding gene in the *C. difficile* genome, a unique allele number was assigned to every sequence variant (Frentrup *et al*., 2020), and allele number 0 was interpreted as absence of gene.

Geographic distances and time intervals

Approximate airline distances between sampling sites were calculated by using the online tool at https://www.mapdevelopers.com/distance_from_to.php. Depending on the information available for database entries, the geographic center of the federal state or country for each sample was used respectively (Suppl. Table S2). When exact sampling dates were not available, approximate sampling time intervals were calculated by using the middle of the sampling year or month respectively (Suppl. Table S2).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Fig. S1. Neighbour-joining tree based on SNP variation among genomes in three HC2 clusters as indicated. Colours indicate isolate sources as in Fig. 3, and the scale bar indicates the branch length corresponding to 1 SNP.

Supplementary Table S1. List of analysed isolates (n = 278). CC, core-genome sequence type complex; HC, hierarchical cluster.

Supplementary Table S2A. Metadata of the 15 external strains.

Supplementary Table S2B. Approximate distances between sampling locations (lower triangle) and time intervals (upper triangle). n.a.: not available.