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Occurrence and characterization of *Clostridioides difficile* in small companion animals and their owners

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A wise man proportions his belief to the evidence.

David Hume

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List of Abbreviations

AAC	antibiotic-associated colitis
AAD	antibiotic-associated diarrhoea
ADR	European Agreement concerning the International Carriage of Dangerous Goods by Road
BMBF	Bundesministerium für Bildung und Forschung (German Federal Ministry of Education and Research)
bp	basepairs
CA-CDI	community-acquired *Clostridioides difficile* infection
C. difficile	*Clostridioides difficile*
CDAD	*Clostridioides difficile*-associated diarrhoea
CDI	*Clostridioides difficile* infection
CDT	CD ADP-ribosyltransferase, meaning the binary toxin of *Clostridioides difficile*
CI	confidence interval
CITP	colonization, infection or transient passage
CPE	cytopathogenic effect
e.g.	"*exempli gratia*" in the sense of "for example"
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EU	European Union
FLI	Friedrich-Loeffler-Institute (Federal Research Institute for Animal Health)
FU	Freie Universität Berlin
GDH	glutamate dehydrogenase
GNC	German National Cohort
GRC	genetically related cluster
HA-CDI	hospital-associated *Clostridioides difficile* infection
ID	identification number
ITS	intergenic transcribed spacer region
MLST	Multilocus sequence typing
MLVA	multilocus variable-number tandem-repeat analysis
n/a	not applicable
NAP	North American pulsed-field
nt	nucleotides
OR	odds ratio(s)
PaLoc	pathogenicity locus
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PMC	pseudomembranous colitis
REA	restriction endonuclease analysis
RFLP	restriction fragment length polymorphism
RKI	Robert Koch Institute
RT(s)	ribotype(s)
SNP(s)	single nucleotide polymorphism(s)
STRD	summed number tandem-repeat differences
TcdA	toxin A
TcdB	toxin B
TMF	Technologie- und Methodenplattform für die vernetzte medizinische Forschung e.V.

UK	United Kingdom
USA	United States of America
VDH	Verband für das Deutsche Hundewesen
WGS	whole genome sequencing
WHO	World Health Organization

1. Introduction

*Clostridioides (*previously *Clostridium) difficile* (*C. difficile*) is considered as a significant pathogen in industrial countries (Crobach et al. 2009), whereas the source of infection is still unclear. In humans the clinical spectrum of *C. difficile* infections (CDI) can vary from light diarrhoea to life-threatening inflammation of the colon (pseudomembranous colitis) (Planche and Arnold 2009). Infections with *C. difficile* are often linked to hospital stays and assumed to be nosocomial infections (Kuijper and van Dissel 2008). Besides, *C. difficile* is among the main infectious agents causing diarrhoea associated with hospitalization and antibiotic treatment. Intensive therapy and prolonged hospitalization due to CDI increase costs in industrial countries, as described in detail for Europe and North-America (Vonberg et al. 2008; Schneider 2007; Kyne et al. 2002). The economic implications caused by CDI are estimated to account for 3 billion Euros per year in the European Union (EU) and 1.1 billion US dollars per year in the United States of America (USA) (Kuijper et al. 2006). One reason for the successful manifestation of *C. difficile* within the healthcare system is the fact that this anaerobic bacterium is able to form endospores which is beneficial for its survival under different environmental conditions (RKI-Ratgeber 2009), therefore, aggravating infection control.

Since 2001 epidemiological changes and the emergence of novel strains causing *C. difficile*-associated diarrhoea (CDAD) have been described worldwide (Rupnik et al. 2009a). Morbidity and mortality seemed to increase in humans and new, more virulent strains have been isolated, in particular ribotype (RT) 027 and RT 078 (Dawson et al. 2009; Freeman et al. 2010; Rupnik et al. 2009a). Besides, patients without obvious risk factors seemed to be more often affected by CDI (CDC Centers for Disease Control and Prevention; 2005; Freeman et al. 2010). However, the reasons for the occurrence of lineages/ribotypes associated with increased disease recurrence and mortality rates along with the global distribution are not yet fully understood (He et al. 2013).

C. difficile can also cause diarrhoea in animals. In addition to wildlife, pets and livestock can also suffer from CDAD (Keel and Songer 2006). Moreover, it is well known that animals, as well as humans, can be asymptomatically colonized and might serve as an infectious source for this pathogen (Keessen et al. 2011b). Apart from colonization or infection, another status and possible reason for faecal shedding of *C. difficile* is the transient passage (Furuya-Kanamori et al. 2015; Ozaki et al. 2004). Indeed, the question whether animals can be infected with human pathogenic *C. difficile* has been discussed for a while. During the last decade, surveys regarding CDI in veterinary medicine have mainly concentrated on livestock, especially large animals, but also on horses. As the contact between humans and small companion animals can be especially intense, in particular regarding dogs and cats, a zoonotic transmission is likely to occur. Yet, up to date there is little evidence to prove or disprove this theory, since little is known about the host spectrum of particular *C. difficile* RTs. Similar to humans, dogs and cats can be affected by CDAD, but *C. difficile* can also be isolated from clinically healthy individuals (Borriello et al. 1983b; Struble et al. 1994). The clinical significance of the detection of *C. difficile* in animals still needs to be unravelled. Accordingly, the knowledge of risk factors associated with infection or colonization in animals is scarce. Even though animals might play a role in human CDI, only few data on the prevalence of *C. difficile* in companion animals and the occurring RTs in Europe, especially in Germany, are available. Studies on the occurrence of human-companion animal-pairs outside of hospital settings resp. without a previous history of CDI are missing so far.

Therefore, the aim of the present thesis was to assess the zoonotic potential of *C. difficile*

within the community. Assuming a close relationship between humans and small companion animals, this survey focused on households shared by animal owners and their dogs and/or cats. Thus, the objectives of this work were to evaluate the occurrence of *C. difficile* in dogs, cats and their owners, to characterize and compare *C. difficile* isolates and to assess risk factors for the acquisition of *C. difficile* and potential transmission routes between small companion animals and their owners.

2. Literature Review

2.1. Taxonomy

C. difficile was discovered in 1935 by Hall and O'Toole and classified as a member of the genus *Bacillus*. Due to the complexity of its isolation and cultivation under anaerobic conditions, it gained the name „*difficilis*", and subsequently became *„difficile"*, meaning "*difficult*". Later it has been reclassified into *Clostridium difficile*. *C. difficile* belongs to the family *Peptostreptococcaceae* within the class *Clostridia* and the phylum *Firmicutes* (Ludwig et al. 2009). All members of the class *Clostridia* are obligate anaerobic, Gram-positive and endospore-forming bacteria. Being an ancient prokaryotic lineage, this class is believed to have evolved 2.34 billion years ago from the bacterial domain, thereby being older than the genus of *Escherichia*, for example (Knight et al. 2015).

Phylogenetic analysis based on the 16S rRNA gene proved a broad diversity within the genus *Clostridium* and the need of a major reclassification (Collins et al. 1994; Lawson et al. 2016) since *C. difficile* belongs to the rRNA cluster XI rather than to cluster I which comprises other representatives of the genus *Clostridium*. Recently, the novel genus *Clostridioides* gen. nov. has been established within the family *Peptostreptococcaceae* and *Clostridium difficile* was reclassified as *Clostridioides difficile* gen. nov. comb. nov. with the type species *Clostridioides difficile* (Lawson et al. 2016). The current reclassification of *C. difficile* by Lawson et al. (2016) has been scientifically acknowledged and is widely accepted within the *C. difficile*-community (Rupnik 2016; Oren and Garrity 2016), in contrast to the previously suggested reclassification into *Peptoclostridium difficile* (Yutin and Galperin 2013; Knight et al. 2015).

2.2. Historical background

In 1935, Hall and O'Toole described the microbiota of new-born, breastfed infants. Thereby, they noticed that the faeces of neonates already comprised a diversity of bacteria that underwent changes during the first ten days after birth. Within the isolated anaerobes a previously not yet described bacillus-like bacterium was detected. In animal experiments the authors showed that guinea pigs and rabbits were highly susceptible for CDI. Interestingly, Hall and O'Toole already suspected the existence of toxins. They assumed that diarrhoea with bloody faeces and inexplicable convulsions in babies might be symptoms of toxin production and persistence of "*Bacillus difficilis"* in the intestinal tract.

Snyder (1937) proved lethal effects of *C. difficile* in various mammals and even birds (guinea pigs, rabbits, pigs, cats, dogs, rats, and pigeons) applying 17 phenotypically similar strains isolated from faeces of 182 infants (two weeks to one year of age) and the strains earlier described by Hall and O'Toole. However, Snyder was not able to detect pathogenic effects if the animals were inoculated orally or directly into the small intestine whereas all subcutaneously infected animals died.

Despite the first descriptions of *C. difficile* by Hall, O'Toole and Snyder and its' lethal effects in animal models, only a few studies followed. Presumably, the lack of continuous research on the newly described bacterium was due to the apparently benign role in the intestinal microbiota of new-borns (Onderdonk and Bartlett 1981). 25 years went by until Smith and King (1962) presented eight clinical human cases. In a guinea pig model all of the eight *C. difficile* isolates detected in different patients proved to be lethal. Smith and King hypothesized, that *C. difficile* is not or if, only rarely, pathogenic in humans. However, in the 70s more researchers started to gain an interest in *C. difficile*, its characteristics and significance as a pathogen. In his PhD dissertation at the University of Leeds, UK, Hafiz

already discovered the occurrence of *C. difficile* in faecal samples from donkeys, horses and camels originating from his native country, Afghanistan (Hafiz 1974). In 1978, George *et al.* identified *C. difficile* as the causative agent of pseudomembranous colitis (PMC) in humans, a disease that had been described for the first time in 1893 by Finney. Moreover, Bartlett et al. (1978) confirmed that the occurrence of PMC caused by *C. difficile* was antibiotic-associated. The association between antibiotic administration and the development of diarrhoea due to *C. difficile* was the first approved risk factor in early risk analysis for CDI (Larson et al. 1978).

Furthermore, Bartlett and colleagues (1978) described cytopathic effects in cytotoxicity assays caused by a *C. difficile* toxin. However, the understanding of toxin production and the mechanisms of toxin action in *C. difficile* remained speculative. Bartlett *et al.* (1980) suspected that *C. difficile* might produce two distinct toxins. Taylor et al. (1981) purified and characterized a toxin which they designated as "toxin A" to differentiate it from the previously described *C. difficile* cytotoxin. Finally, in 1982 Sullivan *et al.* were able to purify and characterize two distinct toxins which can be produced by *C. difficile.* They reported that both toxins had cytotoxic effects and proved their lethality in animal models. "Toxin A" was characterized as an enterotoxin causing fluid accumulation in the infected bowel. The previously called cytotoxin, now termed as "toxin B", was shown to have an even 1,000 to 10,000 higher cytotoxic effect. In addition to toxin A and B, Popoff et al. (1988) later described another virulence factor, an ADP-ribosyltransferase of *C. difficile*, a binary toxin (CDT). Anyhow, the pathogenesis and clinical role of the toxins for the development of PMC and antibiotic-associated colitis (AAC) remained mainly unclear.

Beside the efforts of characterizing virulence factors of *C. difficile*, outbreaks in hospital-settings due to *C. difficile* causing PMC were reported, as early as in the beginnings of the 1980s. Those outbreaks led to the assumption that *C. difficile* acquisition is related to hospitalization, meaning that CDI was a nosocomial infection. The high potential of environmental contamination due to the high persistence of endospores and the transmission from asymptomatic carriers became soon apparent (Fekety et al. 1980). The authors also pointed out that infection and spread from person to person is an important epidemiological factor. Hence, the isolation of infected patients in health-care units was accepted as a simple method to prevent spreading of *C. difficile* (Walters et al. 1982; Fekety et al. 1980; Kim et al. 1983; Rogers et al. 1981).

Thus, rapid and reliable methods for detecting *C. difficile* in hospitalized patients suffering from diarrhoea were required. Viscidi et al. (1983) developed an enzyme-linked immunosorbent assay (ELISA) for detecting antibodies against toxin A and/or B. Later, an ELISA was developed which enabled the detection of toxins A and B in human faecal specimens without using tissue culture assays enabling a rapid diagnosis of CDI in clinical settings (Laughon et al. 1984). Assays detecting toxins A and/or B and/or the antigen glutamate dehydrogenase (GDH) are until now the most widely used diagnostic methods for CDI in clinical laboratories, e.g. in the USA (Kufelnicka and Kirn 2011).

Reviewing the discovery of *C. difficile*, Bartlett (1988) adequately summed up the important milestones of research on this pathogen: (1) the first description of PMC by Finney in 1893, (2) the discovery of *C. difficile* by Hall and O'Toole in 1935, and (3) the initial work on a rodent model for antibiotic-associated colitis in guinea pigs by Hambre *et al.* (1943). Additionally, it has to be mentioned that the detection of toxins was essential to understand the pathogenicity of *C. difficile*.

2.3. Characteristics of *C. difficile* and CDI

2.3.1. Pathogenicity

2.3.1.1. Virulence associated factors

Two large, closely related clostridial proteins, toxin A (TcdA) and B (TcdB), have been identified as primary virulence factors being crucial for *C. difficile*-associated AAD and PMC (Elliott et al. 2017; Bartlett 1990; Rupnik et al. 1997). *C. difficile* is considered to be toxigenic, if at least one of these toxins is present, and is only then potentially able to cause disease. TcdA is referred to as an enterotoxin and TcdB as a cytotoxin; both are glycosyltransferase toxins encoded by the genes *tcdA* and *tcdB* which have a similar size (7 and 8 kb, resp.) and are highly homologous; they are located on the pathogenicity locus or PaLoc (Rupnik et al. 1998; von Eichel-Streiber et al. 1992; Rupnik et al. 2009b). *C. difficile* may also produce a variety of other putative virulence factors, amongst others an actin-specific ADP-ribosyltransferase which was later designated as the third toxin, the binary toxin CDT (cytolethal distending toxin), but also endospores, the surface layer protein complex, cell wall proteins, pili, flagella, and a capsule (Borriello 1990; Borriello et al. 1990; Popoff et al. 1988; Knight et al. 2015; Awad et al. 2014). The binary toxin CDT which is encoded by the *cdtA* and *cdtB* genes with an enzymatic and a binding domain is another factor influencing the pathogenesis and virulence of CDI (Perelle et al. 1997). CDT has been associated with an increased pathogenicity of CDI (Barbut et al. 2005), however, it has not yet been shown that CDT alone can cause clinical symptoms (Geric et al. 2006). There are only few reports on strains harbouring only *tcdB* or *cdtA* and *cdtB* genes (Rupnik et al. 1998; Cairns et al. 2015; Elliott et al. 2009).

Endospores are crucial for acquisition of *C. difficile*, transmission and recurrence of infection (Awad et al. 2014). They are regarded as a dormant form of vegetative *C. difficile* cells highly persistent to environmental stress (aerobic conditions, heat, desiccation, chemicals, disinfectants, and radiation) (Awad et al. 2014; Paredes-Sabja et al. 2014). Essential for spore morphogenesis is a complex assembly of proteins forming the spore cortex and coat; this process is initiated by cell division into a mother cell and a smaller forespore (Putnam et al. 2013; Pettit et al. 2014). SpoIVA and SipL are spore morphogenic proteins involved in the early stages of *C. difficile* coat assembly around the forespore (Putnam et al. 2013). Another factor essentially promoting spore formation is the Spo0A protein, thus, playing a significant role in *C. difficile* host-to-host transmission, persistence and relapsing infections (Deakin et al. 2012; Lawley and Young 2013). The *spo0A* gene has been described as a master regulator also controlling other virulence associated factors such as the production of flagella or influencing metabolic pathways, e.g. by positively regulating the butyrate biosynthetic pathway, thereby, presumably enhancing mucosal adherence (Pettit et al. 2014; Mackin et al. 2013). Additionally, mature spore formation of *C. difficile* is regulated by sporulation sigma factors σ^F, σ^E, σ^G, and/or σ^K (Fimlaid et al. 2013).

The cell surface layer (S-layer) with its S-layer proteins, e.g. SlpA, forms the outer bacterial layer and is involved in cell adhesion and immunogenic reactions inducing inflammatory responses (Awad et al. 2014; Calabi et al. 2002; Ryan et al. 2011; Bianco et al. 2011; Lawley et al. 2009). Furthermore, cell wall proteins of *C. difficile* are believed to promote biofilm-like formation in the host (Reynolds et al. 2011), with other cell wall proteins functioning as tissue degradative enzymes (Rodriguez et al. 2015). Other virulence factors important for the adhesion of *C. difficile* to epithelial cells of the gut are proteins binding to extracellular matrix such as fibronectin, fibrinogen and collagen (Tulli et al. 2013; Cerquetti et al. 2002) and heat shock proteins which are involved in the attachment to eukaryotic cells (Pechine et al. 2013;

Hennequin et al. 2001). Most clostridia possess type IV pili and it has been suggested that type IV pili are also involved in *C. difficile* biofilm formation and play a crucial role in colonization and pathogenicity (Maldarelli et al. 2016; Piepenbrink et al. 2015). In contrast, the role of fimbriae for the virulence of CDI still remains uncertain due to the reason that not all toxigenic *C. difficile* strains seem to be of the need of fimbriae to unfold pathogenic effects in the host (Taha et al. 2007). However, definite virulence factors contributing to the pathogenicity of *C. difficile* are flagella which enable motility, host invasion, and colonization; the latter also facilitated by flagella's involvement in biofilm formation (Awad et al. 2014; Dingle et al. 2011; Baban et al. 2013). A polysaccharide capsule has also been described which inhibits phagocytosis of *C. difficile* (Davies and Borriello 1990; Dailey et al. 1987).

Toxigenic *C. difficile* strains usually harbour a 19.6 kb chromosomal region known as the PaLoc, which comprises of *tcdA* and *tcdB* and three additional genes *tcdR*, *tcdC* and *tcdE* which are involved in the regulation of toxin production (Carter et al. 2011; Mani and Dupuy 2001) or in the extracellular export of the toxin (Govind and Dupuy 2012; Govind et al. 2015). In non-toxigenic strains the PaLoc is replaced by a noncoding region, an integration site (Braun et al. 1996). Yet, *cdtA* and *cdtB* are located apart from the PaLoc on the *C. difficile* chromosome (Perelle et al. 1997).

2.3.1.2. Pathogenesis and human infections

The pathogenic effect of *C. difficile* is usually driven by TcdA and TcdB which are able to inactivate GTPases of the Rho-family by glucosylation. Amongst others, Rho GTPases play an important role by regulating the actin cytoskeleton in cells and controlling the epithelial barrier (Jank and Aktories 2008). Toxins A and B are able to disrupt the actin cytoskeleton resulting in death of enterocytes in the colon and the subsequent destruction of the epithelial barrier and cellular tight junctions (Carter et al. 2012). Hence, apoptosis, cell loss and an inflammatory cascade with release of cytokines as well as invasion of neutrophils and mast cells further compromise the function of the epithelial barrier (Jank et al. 2015; Pothoulakis 2000). Consequently, this results in fluid accumulation in the sore intestine and the manifestation of diarrhoea in the *C. difficile* infected patient (Voth and Ballard 2005).

Recently, it has been proposed that TcdB is of major significance for the virulence of toxigenic *C. difficile* strains (Lyras et al. 2009). Yet, the exact pathogenic pathways, probable synergistic effects of TcdA and TcdB and their mode of action in the cytosol of enterocytes still remain to be unravelled (Jank et al. 2015).

C. difficile infections usually manifest as toxin-mediated intestinal diseases, only rarely, extra-intestinal infections have been reported. Symptoms in affected humans can vary from asymptomatic carriership and mild diarrhoea to more severe abdominal symptoms with pain and fever. Nevertheless, *C. difficile* can also cause severe inflammation of the large intestine with the formation of pseudomembranes (characteristic for PMC), toxic megacolon, sepsis and shock syndrome which can lead to death (Rupnik et al. 2009b).

2.3.2. Molecular typing methods

Epidemiological studies and phylogenetic analysis rely on valid and reproducible molecular techniques for unambiguous characterization of the pathogen. Since the first description of *C. difficile*, a reliable identification of the bacterium has become essential; later, typing methods have been developed to further characterize the diverse pathogen.

2.3.2.1. Restriction endonuclease analysis (REA)

REA is a technique producing banding patterns of chromosomal DNA assigning *C. difficile* strains into different groups, e.g. BI. For this, the extracted total DNA has to be restricted with the frequent-cutting enzyme *Hin*dIII. Resulting fragments are separated by electrophoresis (Rafferty et al. 1998) and the DNA pattern is compared visually to already known REA groups; isolates belonging to the same REA-type have indistinguishable banding patterns with a relatedness of ≥90% (Tenover et al. 2011; Bowman et al. 1991). Although the discriminatory power is thought to be good, the typeability, reproducibility, and interpretability of DNA patterns are only moderately suitable for *C. difficile* characterization (Knetsch et al. 2013; Rafferty et al. 1998; Silva et al. 1994).

2.3.2.2. Pulsed-field gel electrophoresis (PFGE)

The advantage of PFGE is the separation of large DNA fragments during agarose gel electrophoresis using an electrical field of alternating polarity. Prior performing pulsed-field gel electrophoresis, the total genomic DNA has to be digested usually using the restriction enzyme *SmaI* (Killgore et al. 2008). The resulting PFGE-profiles are visualized and analyzed by a computer-assisted tool. (Tenover et al. 2011; Killgore et al. 2008). Although this method is often applied in North American laboratories and isolates are characterized as distinct North American pulsed-field types (NAP-field) (Knetsch et al. 2013; Gerding and Johnson 2013) its discriminatory power for *C. difficile* is considered to be inadequate (Rafferty et al. 1998). Besides a missing common standard protocol, PFGE data are difficult to compare and exchange between laboratories (Dawson et al. 2009).

2.3.2.3. Multilocus sequence typing (MLST)

MLST has become the gold standard to determine the genetic relatedness within a variety of bacterial species (Dawson et al. 2009). For MLST sequencing results of highly conserved regions (alleles) from six to eight housekeeping genes are analysed; the comparison of allelic profiles then allows drawing conclusions on genetic relatedness and population structure. Two different *C. difficile* MLST schemes have been proposed by Lemee and Pons (2010) and Griffiths et al. (2010). The multilocus sequence types (STs) defined on the basis of distinct allel numbers according to the MLST database can be further classified into six distinct clades, however, most STs cluster in clade 1 (Gerding and Johnson 2013; Elliott et al. 2014; Dingle et al. 2014). Although MLST data is easily transferable and reproducible between different laboratories, regarding *C. difficile*, the discriminatory power has been found to be too poor for this bacterial species (Dawson et al. 2009; Knetsch et al. 2013).

2.3.2.4. PCR ribotyping

PCR ribotyping is acknowledged as a reliable, reproducible, simple technique that provides a discriminatory power advantaging other typing methods to characterize and differentiate *C. difficile* isolates (Stubbs et al. 1999; Bidet et al. 2000). Firstly described by Bowman et al. (1991), ribotyping is performed targeting the ribosomal (*rrn*) operon which forms part of the *C. difficile* genome and is present at diverse sites and at a variable number of copies differing between strains of *C. difficile* (Gürtler and Grando 2013). Within all *C. difficile* strains, the *rrn* operon (Figure 1) generally comprises of the 16S rRNA gene, intergenic transcribed spacer region 1 (ITS1; 16S-23S intergenic transcribed spacer region 1), $tRNA^{ala}$ (transfer RNA alanine molecules), 23S rRNA gene, ITS2 (23S-5S intergenic spacer region 2) and 5S rRNA gene.

Figure 1: Ribosomal RNA operon (*rrn*) from *C. difficile*

(figure derived from Gürtler and Grando (2013))

PCR ribotyping amplifies the ITS1 which is located between the 16S rRNA gene and the 23S rRNA gene (Stubbs et al. 1999). Size variations in the ITS1 ranging between 233 basepairs (bp) to 680 bp are used to identify PCR ribotyping patterns (Indra et al. 2008) correlating with *C. difficile* ribotypes (Gürtler and Grando 2013). Later, PCR ribotyping based on capillary gel electrophoresis further enhanced this method (Indra et al. 2008).

According to the *Clostridium difficile* Ribotyping Network (CDRN) more than 600 different RTs of *C. difficile* have been described, so far (Knight et al. 2015). PCR ribotyping has become the standard molecular typing method of choice to characterize *C. difficile* (Rupnik et al. 2001).

2.3.2.5. Toxinotyping (PCR-RFLP)

Toxinotyping is a method to differentiate strains of *C. difficile* according to variations in the coding regions of toxin A and B which form part of the PaLoc; this method is based on amplification of parts of toxin genes and the subsequent digestion of the PCR fragments with restriction enzymes to measure restriction fragment length polymorphisms (RFLP) (Rupnik and Janezic 2016; Rupnik et al. 1998; Rupnik et al. 1997). Genes of the PaLoc are compared to the standard designated as toxinotype "0"; however, the majority of isolates tested so far belong to toxinotype 0 (Gerding and Johnson 2013). Nonetheless, the heterogeneity of *C. difficile* toxin genes currently allows a subdivision into 34 different toxinotypes (I to XXXIV) (Rupnik and Janezic 2016).

2.3.2.6. Multi-Locus Variable-number tandem repeat Analysis (MLVA)

Multi-Locus Variable-number tandem repeat Analysis (MLVA) is a highly discriminatory molecular typing method applied to evaluate the genetic relatedness between *C. difficile* isolates (Marsh et al. 2010). MLVA analyses DNA repeat units at seven tandem-repeat loci via PCR and sizing of PCR products on an automated sequencer and calculates absolute differences in copy numbers for each locus (Eyre et al. 2013b). The calculated numbers of repeats of the seven loci are combined into a string, which is referred to as the MLVA profile. This profile is suitable for comparison and clustering. Hence, MLVA is considered to be a potential tool for epidemiological studies and can be applied to investigate transmission routes in outbreak situations (van den Berg et al. 2007).

2.3.2.7. Whole genome sequencing (WGS)

Whole genome sequencing (WGS) is a method enabling to analyze the gene content and structural variations in specific genes of the accessory genome as well as of the highly conserved core genome which accounts for approx. 80% of the *C. difficile* genome (Eyre et al. 2013b). The analysis of single nucleotide polymorphisms (SNPs) allows the characterization of *C. difficile* isolates and describes phylogenetic relationships on a high discriminatory level (Knetsch et al. 2013; Elliott et al. 2017). This has recently enabled a genome-based tracking revealing the dynamics of CDI in respect of transmission and disease recurrence in patients within a hospital in the UK (Kumar et al. 2016). Currently, MLVA and WGS have been acknowledged to provide the most powerful data to study epidemiological aspects of *C. difficile* strains and CDI (Knight et al. 2015). Yet, the comparison of obtained *C. difficile* whole genome data is still impaired due to the fact that only a few "fully closed" high quality

genomes are available, so far (Knight et al. 2015).

2.3.3. Epidemiological changes

Since the first description of *C. difficile* in 1935, the knowledge of its existence and its potential pathogenicity was restricted to a small circle of scientists. While in the 1960s it was still contemplated that *C. difficile* might not be pathogenic for human hosts (Smith and King 1962), in the 1970s infections in humans were more frequently reported, especially cases of PMC. Additionally, the first cases of antibiotic-associated diarrhoea were associated with *C. difficile* (Bartlett et al. 1978; George et al. 1978). During the following years increased infection rates due to *C. difficile* were reported and detection methods improved (George et al. 1979; Kim et al. 1983). Hospitalization, advanced age and antibiotic treatment were identified as potential risk factors for infections in humans (Rupnik et al. 2009b). Yet, the rise in CDI incidence is nowadays reflected as a result of improved diagnostic methods and the awoken awareness rather than a genuine explosion of human infections (Freeman et al. 2010). Although the increased incidence should be carefully evaluated, the emergence of novel RTs described as hypervirulent had a huge impact in the new millennium (Freeman et al. 2010; Goorhuis et al. 2008a).

Since the beginning of the new millennium, immense nosocomial CDI outbreaks have been reported in the USA, and later also in Europe, Asia, Australia and Latin America (Dawson et al. 2009; Freeman et al. 2010; Rupnik et al. 2009b; Clements et al. 2010; Aguayo et al. 2015). The responsible strain was described as NAP 1, REA group BI and PCR RT 027 (sometimes referred to as "BI/NAP1/027" (Freeman et al. 2010)), positive for toxins A and B and the binary toxin CDT. RT 027 has been linked with an increase in the severity and frequency of clinical infections as well as higher mortality and recurrence rates in several countries (Hubert et al. 2007; Goorhuis et al. 2007; Sundram et al. 2009). Although RT 027 had already been isolated in 1984, it had been described as non-epidemic at that time; the sudden epidemic character of novel RT 027 strains was presumably a result of the excessive use of fluoroquinolones (McDonald et al. 2005). Due to reduced susceptibility, e.g. to newer fluoroquinolones based on acquired mutations in the genes *gyrA* and *gyrB*, therapeutic options were often limited in case of *C. difficile* infections with the novel RT 027 (Drudy et al. 2007; Dridi et al. 2002). Moreover, they cause more complicated CDAD with increased recurrence rates (Petrella et al. 2012). According to an European hospital-based survey 5% of the *C. difficile* infections in hospitalized patients were caused by RT 027 (Bauer et al. 2011). RT 027 strains of an international *C. difficile* animal strain collection originated from bovine hosts only (Janezic et al. 2014). Thus, RT 027 is mainly considered as a predominant human strain which seems to be of minor relevance for animal hosts (Janezic et al. 2012; Freeman et al. 2010).

Another new emerging *C. difficile* strain was RT 078 which has been linked to community-associated CDAD. RT 078 is a toxigenic strain encoding for toxin genes *tcdA*, *tcdB* and the binary toxin genes as well; it has been assigned to toxinotype V and the PFGE type NAP7 (Tenover et al. 2011; Debast et al. 2009). In a European hospital survey in 2007, Barbut and his coworkers found that RT 078 was the 11th most prevalent *C. difficile* strain, only dominating in Greece. Though the study design differed, Bauer et al. (2011) described RT 078 as the third most prevalent isolate identified in 18 different countries in a hospital-based European survey, only 4 years later. Similar results had been reported from the USA (Rupnik et al. 2008). Compared to RT 027, RT 078 is more often related with community-acquired *C. difficile* infections (CA-CDI) (see 2.3.3.1) and affected patients tend to be younger (Jones et al. 2013; Keessen et al. 2011b). Also, RT 078 is the predominant RT in bovine and porcine

faecal samples, isolated in 94% (31/33) and 83% (119/144) of calf and piglet samples (Keel et al. 2007). In contrast to RT 027, where a decrease in incidence has been described after the implementation of hospital surveillance programs (Hensgens et al. 2009), the incidence of RT 078-infections seems to increase, e.g. from 3% of all isolates from CDI-patients in 2005 to 13% in 2007 in the Netherlands (Goorhuis et al. 2008a). The authors pointed out that RTs 027 and 078 have a similar clinical impact regarding the severity of CDI and mortality rates. However, RT 078 was less frequently associated with epidemic nosocomial outbreaks (Freeman et al. 2010).

Recently, it has been proposed that the rise of CDI outbreaks since the beginning of the 2000s might be linked to epidemic RTs 027 and 078 having acquired the ability to grow on unusually low concentrations of disaccharide trehalose in contrast to other *C. difficile* strains (Collins et al. 2018). Trehalose is a carbohydrate food additive enriching food products for human diet, e.g. pasta, ice cream, and ground beef; since 2001 trehalose production increased considerably (Ballard 2018; Higashiyama 2002) which coincides with the occurrence of epidemic CDI outbreaks caused by RT 027 and 078 (Collins et al. 2018). Collins et al. pointed out that both RTs belong to phylogenetically distant clades and have acquired different mechanisms to grow on low levels of trehalose. In RT 027 a mutation of the protein TreR increases the affinity of the protein for trehalose. In contrast, RT 078 possesses the transporter PtsT located on its membrane leading trehalose into the cell. In a murine model the authors also proofed that dietary trehalose addition increased disease severity and mortality, presumably, due to elevated levels of toxin production. Hence, RT 027 and 078 benefit from a competitive advantage in fitness due to the ability to metabolize lower trehalose levels.

Reviewing the new developments in epidemiology and pathology Rupnik et al. (2009) emphasized that, although hospitalized patients of advanced age receiving antibiotics are still regarded as the highest risk group of CDI, infections in people considered to be at low risks like younger patients, pregnant women and children without previous hospitalization and antibiotic treatment have increased. Regarding the emergence of novel toxigenic RTs 027 and 078, Rupnik (2007) retrospectively reviewed the occurrence of the binary toxin CDT. The study revealed that strains harbouring *cdtA* and *cdtB* genes were formerly rarely isolated in humans, with approximately 2 to 10% in a non-outbreak situation. In contrast, isolates positive for the binary toxin were isolated in 43 up to 100% from different animal species, with horses harbouring the lowest number (up to 43%) and cattle the highest (up to 100%). Rupnik assumed that early studies indicated that two distinct lineages of *C. difficile* existed with animals being more prone to harbour *cdtA*/*cdtB*-positive strains than humans. This has changed during the last two decades as the prevalence for CDT-producing strains increased in humans. Changes in human *C. difficile* isolates were clearly demonstrated in an Italian survey which compared human strains isolated during three different time periods (before 1990, 1991-1999 and 2000-2001). The authors found a rising number of binary toxin producing isolates from 0% and 24% up to 45% during this time (Spigaglia and Mastrantonio 2004). Moreover, the strains encoding genes for the binary toxin were more often associated with severe CA-CDI (Barbut et al. 2005).

2.3.3.1. Community-acquired *C. difficile* infections

Apart from hospital-associated CDI (HA-CDI), the increasing incidence of CDI-cases outside of healthcare facilities made it imperative to identify the infection source. For distinction of infections not associated with healthcare settings a definition was required; hence, an *ad hoc* surveillance group for *C. difficile* offered an accepted designation (Freeman et al. 2010; Keessen and Lipman 2012). Thus, CDI was defined as a community-acquired infection with *C. difficile* in a patient who had not been discharged from healthcare in a twelve week period

before an onset of disease occurring within the community or maximally 48 hours after admission to a healthcare facility (McDonald et al. 2007; Kuijper et al. 2006). CDI-cases occurring between four and twelve weeks after discharge from a healthcare facility may be community- or healthcare-associated, depending on the case's history; this group is characterized by a community-onset of CDI and a previous hospital stay at least four weeks before disease onset making it difficult to differentiate between HA- and CA-CDI (Kuijper et al. 2006).

With the emergence of RTs 027 and 078 and the subsequent changes in epidemiology the research focus increasingly shifted to other potential infectious sources and risk factors. So, Hensgens et al. (2012) pointed out that the source of infection for patients with diagnosed CA-CDI must, by definition, lie outside of hospital settings. Whether animals play a significant role in direct or indirect transmission and represent an infectious reservoir for *C. difficile* remains to be unravelled. This was an aim of my thesis.

2.4. *C. difficile* in veterinary medicine

2.4.1. Historical retrospection

Although veterinary research on *C. difficile* focussed mainly on animal model systems applying laboratory animals, like mice (*Mus musculus*), rats (*Rattus norvegicus* and *Rattus rattus*), Syrian hamsters (*Mesocricetus auratus*), guinea pigs (*Cavia porcellus*), and rabbits (*Oryctolagus cuniculus*), its detection in faecal specimens of other species has also been increasingly reported. McBee (1960) described *C. difficile* in the intestinal microbiota of Antarctic birds and Weddell seals. Moreover, Small (1968) very early described fatal enteric disease in laboratory hamsters after lincomycin administration, nowadays often cited as the first report on AAC.

However, the first isolation of *C. difficile* from non-laboratory, domestic animals was described in a goat by Hunter et al. (1981). Pathologic and microbiologic examination revealed multiple abscesses in the mandible of this goat with *C. difficile* being identified in pure culture. Later, the examination of bovine faeces for clostridial species in Nigerian livestock resulted in the detection of *C. difficile* (Princewell and Agba 1982). The first report of *C. difficile* infected pigs followed in 1983 (Jones and Hunter 1983), although, the pathogen had already previously been reported to cause dysentery in experimentally infected gnotobiotic pigs (Lysons et al. 1980). Besides, Borriello et al. (1982) showed that a variety of mammalian (cattle, goat, dogs, cats and hedgehog) and avian species (ducks, geese, chicken and ring-necked parakeet) can also harbour *C. difficile.* Interestingly, the authors hypothesized that a susceptible host mostly acquires *C. difficile* from the environment in hospital- or community-settings.

Moreover, a case of AAC with detection of *C. difficile* in an Alaskan brown bear held in captivity was reported (Orchard et al. 1983). The bear suffered from enterocolitis after being treated with antibiotics for an abscess in the lumbosacral region. By means of colonoscopy, ulcers described as pseudomembranes were diagnosed typical for a *C. difficile* colitis in humans.

2.4.2. *C. difficile* in horses

As early as in 1981 a lincomycin associated fatal enterocolitis had been described in horses for the first time (Raisbeck et al. 1981). However, detailed clinical examinations of the diarrhoeic horses followed by necropsy, chemical and microbiological analysis could not reveal a bacteriologic agent. The authors suspected a toxic dose of lincomycin due to accidental feed contamination causing the fatal diarrhoea in the horses. Further clinical reports

in equine patients followed, with Jones et al. (1987) reporting on the presence of *C. difficile* in foals that had not received antibiotics before the onset of diarrhoea. 63% (27/43) of the diarrhoeic foals were positive for *C. difficile* but none of the faecal samples from asymptomatic foals or healthy adult horses. Jones pointed out that the distinct association between *C. difficile* isolation and diarrhoea in foals contradicted the findings in human infants who are often asymptomatic carriers of *C. difficile*. Later, Jones et al. (1988) linked the deaths

of four new-born foals to CDI; the foals had suffered from haemorrhagic necrotizing enterocolitis with abdominal cramping and diarrhoea resembling symptoms of severe CDAD cases in humans. *C. difficile* was subsequently recognized as a potential pathogen causing enterocolitis in foals (Jones 1989). The authors presumed that *C. difficile* as a pathogen might have been underestimated in the past due to scarce knowledge about this anaerobic bacterium and it's challenging isolation and identification. Jones and his colleagues pointed out the necessity for both cytotoxin testing as well as isolation of *C. difficile* in foals. Later, Fey and Sasse (1997) reviewed case reports describing the impact of antibiotics on the gut microbiota of horses. Their meta-analysis showed that antibiotic-associated diarrhoea (AAD) in horses is often accompanied with proliferation of clostridial species with progressing rise of coliform and streptococcal bacteria. However, the authors emphasized that research on CDI in humans as well as in equine patients should focus on comparative characterization of *C. difficile* in asymptomatic and infected horses to identify distinct virulence factors.

Similar to the findings in younger children, Baverud et al. (2003) described healthy foals younger than 14 days asymptomatically carrying toxigenic strains. Consequently, foals were suspected to be a potential source of infection for other horses. Moreover, as it has been shown that the pathogen can survive in the environment for at least four years, asymptomatic shedding of *C. difficile* could be an underestimated risk (Baverud et al. 2003). The authors also confirmed that *C. difficile*-positivity was associated with AAD in adult horses. Besides, Greiss et al. (1996) and Perk et al. (1993) described that CDI in horses can also present as typhlocolitis with common colic symptoms, typically associated with a significant increase in anaerobic bacteria in faeces (Greiss et al. 1996).

In 1995, the first outbreak of CDAD in an equine veterinary teaching hospital was reported with 9 out of 10 horses harbouring toxigenic *C. difficile* strains (Madewell et al. 1995). All affected horses were under antibiotic treatment at the time of the outbreak. Similar to the findings in human medicine, antibiotic intake became a widely acknowledged risk factor for CDI in horses (Baverud et al. 2003; Greiss et al. 1996; Weese et al. 2000b; Baverud et al. 1997). Moreover, the association between CDI and hospitalization has suggested that *C. difficile* infection in horses might also often be of nosocomial origin similar to CDI in man (Baverud et al. 1998). Nevertheless, further research confirmed that not only nosocomial infections due to *C. difficile* seem to affect horses but also CDAD acquired outside of veterinary hospital settings (Weese et al. 2006).

2.4.3. *C. difficile* in small companion animals

Considering the intense contact between companion animals and their owners, they have been suspected to play an important role in the epidemiology of human CDI. Borriello et al. (1982); (1983a) showed that *C. difficile* carriage in dogs, cats and avian pets is likely to occur, but there was usually no significant association with gastrointestinal diseases or previous antibiotic treatment. In contrast, Berry and Levett (1986) described three cases of chronic diarrhoea apparently caused by *C. difficile* in dogs. That was the first report on clinical CDI in domestic dogs. Moreover, antibiotic treatment of prairie dogs with a second generation cephalosporin (cefoxitin) caused diarrhoea (Muller et al. 1987). Similar to human CDI, the infected prairie dogs had developed pseudomembranes in the caeca; hence, the authors

assumed the suitability as an animal model for further research about CDI in mammals.

In contrast to human enteric symptoms due to CDI, they seem to be less common in dogs and cats (Weber et al. 1989, 1988). Nevertheless, the authors stated that dogs and cats can carry and shed *C. difficile* and might therefore play an important role in the epidemiology of human CDI. Similarly, Riley et al. (1991) described a significant gastrointestinal *C. difficile* carriage rate of up to 39.5% (32/81) in dogs and cats which was later confirmed by O´Neill et al. (1993). Riley and his colleagues reiterated that companion animals are likely to be a significant source of infection. Moreover, they detected high levels of environmental contamination on a variety of different surfaces in two veterinary clinics. O´Neill et al. (1993) concluded that companion animals shedding *C. difficile* might serve as a significant factor in the epidemiology of CDI since it was generally accepted that most patients suffering from CDAD might become infected through environmental sources. Nevertheless, O'Neill *et al.* were not able to prove an overlap between *C. difficile* strains isolated from pets and humans using REA and RFLP. However, analyzing human and dog isolates by PCR ribotyping enabled the identification of the predominant, toxigenic, canine RT denominated as RT A which was also found in 20% (4/20) of the human isolates (Arroyo et al. 2005a). While this finding did not prove interspecies transmission it showed that human and small companion animals can share same *C. difficile* types.

Similar to the findings in humans and new-born foals neonate dogs could be infected or colonized without showing any clinical symptoms (Perrin et al. 1993; Álvarez-Pérez et al. 2015). Both surveys proved that transient infections can occur in puppy dogs during a 3- to 10-week period. The study design comprised weekly faecal examination. The carriage rate ranged from 3 (1/31) to 67% (47/70) (Perrin et al. 1993) and 0 to 100% (18/18) (Álvarez-Pérez et al. 2015). Monitoring 70 puppies and their dams belonging to 14 litters Perrin et al. observed highest carriage rates during the second and third week, which decreased from then on progressively to 3%. Within the same survey, 74 healthy dogs, aged 3 months or older, represented the control group and had a low prevalence of 1.4% (1/74). However, during the first 10 weeks of the puppies' lives 94% (66/70) of them were at least once positive for *C. difficile*, while only 43% (6/14) of the 14 dams were positive at the same time. The high isolation rates in neonate dogs are comparable to those in human infants (Aljumaili et al. 1984; Wendt et al. 2014). Interestingly, toxigenic and non-toxigenic *C. difficile* strains could be found at the same time in puppies and dams of the same litter (Perrin et al. 1993). Furthermore, the authors observed that in twelve transiently infected puppies toxigenic and non-toxigenic strains alternated.

The first nosocomial outbreak of CDAD in a small animal clinic was reported in 2003 (Weese and Armstrong). 52% (48/93) of the tested dogs were positive for toxigenic *C. difficile* during 5 months. All 93 dogs were client or resident dogs and suffered from diarrhoea. It was suggested that environmental contamination was mainly responsible for the outbreak because no direct animal-to-animal-contact had taken place. After the implementation of an effective disinfection and hygiene management the number of CDAD cases decreased significantly. However, the differentiation between hospital- or community-acquired CDI was not possible. Besides, the authors pointed out that *C. difficile* might be an underestimated pathogen in small companion animals and recommended to include screening for *C. difficile* toxins in standard testing protocols.

The *C. difficile* detection rate in diarrhoeic dogs ranged from 1% to 76% (Perrin et al. 1993; Weber et al. 1989; Chouicha and Marks 2006; Silva et al. 2013b). Diarrhoeic dogs had a five-time increased chance to carry *C. difficile* compared to non-diarrhoeic individuals (Diniz et al. 2018). Yet, a recent survey could not confirm an association between diarrhoea and *C.*

difficile detection in dogs attending veterinary clinics (Álvarez-Pérez et al. 2017b). Struble et al. (1994) reported that only 36% (5/14) of the animals carrying a toxigenic *C. difficile* strain also suffered from diarrhoea. The authors concluded that asymptomatic colonization with toxigenic *C. difficile* strains occurs more often in animals than in humans. In Japan, Usui et al. (2016) found that 30% (62/204) of non-diarrhoeic dogs harboured *C. difficile* with 47% (32/68) of the isolated strains producing toxins A and B. While Marks et al. (2002) described that diarrhoea was associated with the detection of toxin A they could not show that the isolation of *C. difficile* (toxigenic and non-toxigenic strains) was linked to diarrhoea. Corresponding to this finding, Clooten et al. (2003) described that they were not able to fulfil Koch's postulates in an animal model inoculating healthy dogs with toxigenic *C. difficile*. None of the dogs showed any symptoms of CDI and *C. difficile* could only be isolated in the faeces from two out of six infected dogs. Those findings further questioned the role of *C. difficile* as a primary enteropathogen in companion animals.

Furthermore, 58% (59/102) of healthy dogs visiting hospitalized patients were positive for *C. difficile* (Lefebvre et al. 2006b). Also, 71% (41/58) of the isolates proved to be toxigenic. One of the tested dogs harboured the epidemic strain RT 027. In 2009, Lefebvre & Weese isolated a second RT 027 strain again from a hospital visitation dog. These two cases have been the only descriptions of RT 027 in small companion animals, so far. Both canine isolates harboured not only toxins A and B but also the binary toxin CDT, although the dogs were asymptomatic carriers.

2.4.4. *C. difficile* in food-producing animals (except poultry)

In 1980, Lysons *et al.* reported on gnotobiotic pigs that had "accidentally" been infected with *C. difficile* in an experiment when studying the role of *Treponema hyodysenteriae* in swine dysentery. Although *C. difficile* increased the severity of clinical symptoms and intestinal lesions, faecal samples from farm pigs suffering from acute swine dysentery and from healthy pigs did not affirm *C. difficile* as a pathogen in pigs.

The first isolation of *C. difficile* in domestic pigs suffering from enterocolitis was reported by Jones and Hunter (1983). Although *Salmonella* Typhimurium had also been identified in the affected pigs, they suspected *C. difficile* as a potential porcine pathogen. Interestingly, the effect of oral antibiotic growth promoters disrupting the physiologic intestinal microbiota had already been considered as a predisposing factor for enterocolitis in pigs (Jones et al. 1987). Nonetheless, 15 years went by until Waters et al. (1998) reported a CDI outbreak in suckling piglets which were diagnosed with typhlocolitis and an estimated mortality rate of 90%.

Songer (2004) identified CDI as the probably most important reason for diarrhoea in neonatal pigs. Clinical symptoms were often associated with antibiotic administration in piglets (Yaeger et al. 2002; Schneeberg et al. 2013a). Routine application of antibiotics and environmental distress were found to foster infections with *C. difficile* and increase mortality in periparturient sows (Kiss and Bilkei 2005). Evidently, cessation of antibiotic administration decreased sow mortality in a porcine outdoor production (Kiss and Bilkei 2005). As neonatal piglets in the same outdoor production line were not affected the authors concluded that the outbreak was not due to an increased environmental contamination but that the sows harboured *C. difficile* before the onset of clinical symptoms. Thus, stress and antibiotic intake might have led to CDAD.

The first isolation of *C. difficile* from the rumen of neonatal lambs was reported by Rieu-Lesme (1999). The authors suggested that the rumen might represent a potential source of infection for humans and pose a significant risk due to environmental contamination. Neonatal calves also harboured *C. difficile* with a prevalence up to 51% (88/172) which was

recorded one week after arrival (Costa et al. 2011). The authors reported an age-dependent prevalence with 32% (56/174) *C. difficile*-carrying calves at the age of <48 hours decreasing significantly to 2% in 21 weeks-old calves. Monitoring 50 veal calves in the US in a 4-6 week interval from the age of approximately one week up to the time of slaughter over a period of 18-22 weeks, Houser et al. (2012) reported that 28% (56/200) of the calves were at least once positive for *C. difficile.* Interestingly, the authors detected the highest shedding rate with 12% (18/150) directly before slaughter and concluded that this could have an impact on ground veal products intended for human consumption. In contrast, the results of a longitudinal survey conducted in Italy by Magistrali et al. (2015) demonstrated that the younger the veal calves were the more likely they shed *C. difficile* with isolation rates of 20.2% (85/420) for 13-28 days old veal calves, decreasing to 0% for 150 days old calves or at the slaughterhouse. Similar results had also been previously described by Rodriguez-Palacios et al. (2006). Costa et al. (2012) assumed that the innate intestinal microbiota as well as the naïve immune system and management factors, like post-natal stress or feed, could facilitate colonization with *C. difficile* in younger individuals.

The question whether *C. difficile* is a primary pathogen in the bovine host has been discussed controversially. Magistrali et al. (2015) confirmed an association between diarrhoea and *C. difficile* shedding. Though, enteric disease was not significantly associated with *C. difficile*-positivity in an earlier study (Rodriguez-Palacios et al. 2006); also, Hammitt et al. (2008) found 30% (16/53) of non-diarrhoeic calves being positive for toxins A and B. Thus, Hammitt et al. (2008) concluded that calves do, at least, serve as hosts in which the agent can multiply. However, Rodriguez-Palacios et al. (2006) identified toxigenic *C. difficile* isolates in calves with RTs of typical epidemic outbreak strains in human hospitals, i.e. RT 017 and 027. Furthermore, the toxigenic RT 078 often discussed as highly pathogenic was predominant in neonatal calves and piglets, detected in 94% (31/33) and 83% (119/144) of the isolates (Keel et al. 2007). Although the samples were collected in different geographic regions of the United States and the animal populations had no contact to each other, the heterogeneity of porcine and bovine RTs was scarce; suggesting that RT 078 circulates independently within those animal populations. Goorhuis et al. (2008b) and Rupnik et al. (2008) found that RT 078 was also one of the most prevalent *C. difficile* strains in humans causing CA-CDI in the Netherlands and the US. RT 078 has later been associated with CDAD in pigs and farmers proving that human and porcine isolates were genetically highly related (Keessen et al. 2013; Knight et al. 2015). Moreover, RT 014/0 which was the most prevalent *C. difficile* strain in a European hospital-based survey was also detected in calves (Bauer et al. 2011). Finally, due to the fact that *C. difficile* isolates from calves and humans belonged to identical genetic lineages, Rodriguez-Palacios and his co-workers (2006) suspected that zoonotic transmission might be probable.

In addition, MLVA revealed that human and porcine RT 078 isolates were genetically highly related (Debast et al. 2009; Bakker et al. 2010). Knetsch et al. (2014) sequenced the genome of 65 *C. difficile* RT 078 strains. All strains were collected in the Netherlands between 2002 and 2011 and originated from pigs, asymptomatically colonized farmers and hospitalized human patients. The WGS based analysis revealed that clonality of RT 078 pig-farmer isolate pairs was common. The authors concluded that transmission may occur from pigs to farmers, especially via faecal-oral infection-route; they also assumed that no interspecies barrier might exist for RT 078. Recently, 247 RT 078 strains originating from animals (livestock) and humans from 22 Asian, Australian, European, and North American countries were compared using WGS (Knetsch et al. 2018). The analyses proofed that RT 078 is characterized by a high clonality, although it seems to be frequently transmitted between continents and farm animals and humans; additionally, diverse antimicrobial resistance genes are carried on

mobile genetic elements being also transmitted between species. Knetsch et al. (2018) gave emphasis to the significance of RT 078 in the context of One Health: the zoonotic transfer of *C. difficile* must be taken into account by infection control measures.

Interestingly, the *C. difficile* prevalence in goats and sheep seems to be lower than in other food-producing animals, though, studies concerning *C. difficile* in small ruminants are scarce. The isolation rates in sheep varied from 0% (0/57) in the US (McNamara et al. 2011), 1% (1/100) in the UK (AlSaif and Brazier 1996), up to 18.2% (2/11) in the Netherlands (Koene et al. 2012). Knight and Riley (2013) explained the low *C. difficile* prevalence with 0.6% (1/156) in sheep and 6.5% (14/215) in lambs with the low exposure of these animals to antimicrobials. Based on the low prevalence rates, Squire et al. (2015) suspected that sheep pose a low risk for *C. difficile* spillover from animals to humans.

2.4.4.1. *C. difficile* in birds

C. difficile can be isolated from various avian species as well. 29% (29/100) of faecal samples from broiler chickens were positive for *C. difficile* and 90% of those isolates were toxigenic (Simango and Mwakurudza 2008). However, investigations on *C. difficile* in poultry have been scarce for a long time, and the most common enteric pathogen in poultry is *Clostridium perfringens* (Cooper et al. 2013). Zidaric et al. (2008) published first European data on the occurrence and characterization of *C. difficile* in poultry. The authors described a significant age-dependent isolation rate for *C. difficile* in laying hens with no apparent enteric disorders. Colonization rates in faecal samples from 2-weeks-old chicks accounted for 100% (24/24) and decreased to 71% (5/7) and 41% (9/22) in 14-weeks- and 18-weeks-old birds, respectively. Thereby, 44 isolated strains belonged to 12 different RTs displaying an unexpectedly high diversity compared to other species. However, the isolation rate in faecal samples from 42-day-old broiler chickens in Texas was significantly lower with 2.3% (7/300) (Harvey et al. 2011) than the overall isolation rate of 62% (38/61) described by Zidaric et al. (2008).

Migratory birds can harbour a diversity of pathogens including clostridial species, and are potential vectors for transmission and spread between distant regions, even continents (Altizer et al. 2011; Hubálek 2004). Hence, Bandelj et al. (2011) collected samples of wild passerine birds belonging to six different species. The birds had hatched in Europe and were sampled in Slovenia on their first migration route to Southern Europe and Africa; thus, the sampled birds were mostly juvenile. According to the results of Zidaric et al. (2008) Bandelj *et al.* expected a high prevalence of *C. difficile* in their study population. Additionally, Zidaric et al. (2010) had reported that 60% (42/69) of water samples from rivers in Slovenia were positive for *C. difficile* with positive sampling sites correlating with increased population density. Therefore rivers were thought to be a possible source of infection for wild birds. However, despite approved detection methods Bandelj and her colleagues were not able to isolate *C. difficile* from young migrating passerines. The authors concluded that wild migrating passerine birds are unlikely to serve as reservoirs for *C. difficile* but assumed that this might be different for birds with a closer contact to humans and their habitats.

Consequently, Bandelj et al. (2014) sampled European barn swallows (*Hirundo rustica*) in autumn on their migration to Africa in a congregation area in Slovenia. Barn swallows are

known to nest in close proximity to human habitats including farms (Møller 2001). Bandelj and colleagues were able to detect *C. difficile* in 4% (7/175) of the sampled barn swallows. All isolates originated from juvenile birds (7/152) and no adult bird (0/23) harboured *C. difficile*, which supports the findings of Zidaric et al. (2008). Interestingly, most of the strains belonged to toxigenic RTs also associated with CDI in humans, like RTs 078 and 014/0. The

authors assumed that barn swallows could rather be indicators for environmental contamination than a source of infection and spread for *C. difficile*.

2.4.4.2. *C. difficile* in exotic animals

C. difficile also caused infections in wild animals held in captivity, like Kodiak bears (*Ursus arctos*) (Orchard et al. 1983), penguins (*Eudyptes chrysolophus*) (Hines and Dickerson 1993), different species of nonhuman primates (*Macaca mara, Hylobates concolor, Cercopithecus diana* (Meisel-Mikolajczyk et al. 1997) and *Saguinus oedipus* (Rolland et al. 1997)), lions (*Panthera leo*) (Meisel-Mikolajczyk et al. 1997), elks (*Cervus elaphus*) (Arroyo et al. 2005b), and Asian elephants (*Elephas maximus*) (Bojesen et al. 2006). Frazier et al. (1993) described *C. difficile* as the causative agent of an outbreak of necrotizing enteritis with acute deaths in a group of captive ostrich chicks (*Struthio camelus*). Álvarez-Pérez et al. (2014) reported low overall *C. difficile* prevalence (3.5 %) in 200 samples of 40 different species in a Spanish zoo. The isolates originated from a chimpanzee (*Pan troglodytes troglodytes*), two goats (*Capra hircus* and *Capra pyrenaica hispanica*) and three zebras (*Equus quagga burchellii*). Interestingly, 57% (4/7) of the isolates belonged to the hypervirulent RT 078. Additionally, *C. difficile* has been isolated from marine molluscs, fish and crustaceans posing a potential risk for human health (Metcalf et al. 2011; Troiano et al. 2015).

A case of CDAD in a wild ocelot (*Leopardus pardalis*) following antibiotic treatment, after being run over and administered to a veterinary hospital in Brazil, was reported by Silva et al. (2013a). The *C. difficile* isolate was positive for toxin genes *tcdA* and *tcdB*; however, the RT had not been identified.

Interestingly, the consumption of large amounts of broccoli was suspected to have triggered an outbreak of CDI in a small herd of five Asian elephants kept in a zoological garden in Denmark (Bojesen et al. 2006). No antimicrobial treatment and no health compromising influence preceded the outbreak causing fatal enterocolitis in two of the infected elephants. It was concluded that the impact of considerable quantities of sulforaphane, a molecule highly present in cruciferous plants (Donaldson 2004), might have destroyed the gut microbiotia due

to its antimicrobial effects. Hence, it was proposed that the ingestion of sulforaphane could have had the same effect as antibiotics by altering the physiological intestinal microbiota and thus, triggering the overgrowth of *C. difficile* (Bojesen et al. 2006).

2.4.4.3. *C. difficile* in wild urban animals

Wild animals occupying urban areas (e.g. due to habitat loss) can also harbour toxigenic *C. difficile* RTs associated with CDI in humans. Silva et al. (2014) described the prevalence of *C. difficile* in 46 free-living South American coatis (*Nasua nasua*). This species lives in forests of close human proximity and ranges between wild and domestic areas (Rodrigues et al. 2006). All animals appeared healthy, and were sampled after being trapped. Although the *C. difficile* isolation rate of 6.5% (3/46) was relatively low, toxigenic RTs 014/020 and 106 were isolated which are associated with CDI in humans (Freeman et al. 2010; Silva et al. 2014).

In California more than 240 individuals of a protected sea otter species (*Enhydra lutris nereis*) were sampled for enteric bacterial pathogens (Miller et al. 2010). Faecal contamination of marine habitats is a health risk for marine mammals becoming infected with pathogens. This can lead to high morbidity and mortality rates, e.g. in sea otters occupying urbanized coastlines. *C. difficile* was identified amongst the isolated bacteria in 6% (12/194) of the faecal samples, with a significantly higher prevalence of 8% (8/98) in deceased sea otters. The authors proposed that this might be due to severe health implications in the dead otters and/or bacterial overgrowth in the *post-mortem* animal.

In a Canadian survey small and medium-sized wild mammals were sampled on different livestock farms (Jardine et al. 2013). *C. difficile* was isolated in 4.6% (5/109) with positive samples from raccoons (*Procyon lotor*) and a shrew (*Blarina brevicauda*). All isolates were toxigenic and belonged to different RTs but none could be designated to one of the over 3,000 human and animal *C. difficile* isolates present in the authors' library. Surprisingly, none of the isolates was identified as RT 078 which is the predominating RT in livestock (Goorhuis et al. 2008b; Weese et al. 2010c). This led to the assumption that wild mammals might not represent a reservoir for *C. difficile* strains which cause infections in humans, companion animals or livestock. Furthermore, wild mammals did not seem to be involved in transmission of *C. difficile* between farms. Hence, Jardine et al. (2013) hypothesized that wild-life could harbour host-adapted *C. difficile* strains.

In contrast, Burt et al. (2012) and Himsworth et al. (2014) isolated pathogenic *C. difficile* from house mice (*Mus musculus*) and rats (*Rattus norvegicus* and *Rattus rattus*). Himsworth et al. (2014) proposed that wild urban animals might play a, so far, underestimated role in transmission and spread of the pathogen. The isolation rate for *C. difficile* in wild urban Norway (*Rattus norvegicus*) and black rats (*Rattus rattus*) in Vancouver, Canada, was 13% (95/724). All 95 isolates were toxigenic and belonged to 35 different RTs. 24 of the isolates had been identified as RTs, which also cause human CDI, such as RT 027, 078 or 014/0. Since the occurrence of *C. difficile* was geographically linked to certain city blocks but without specific RT clustering, Himsworth and colleagues suggested that transmission of *C. difficile* among rats is negligible, even though urban rats live in close-knit communities. Rats rather appear to acquire the pathogen due to environmental contamination and may serve as indicator animals or sentinel species. The authors assumed that direct contact to human excrements could have been the source of infection for rats. Additionally, Himsworth et al. speculated about other potential sources for *C. difficile* contamination in rats, such as contact to the sewage system, access to facilities handling livestock-associated food products, and the direct contact to companion animals, like dogs and cats. Moreover, they concluded that (1) *C. difficile* does not cause infection in rats; (2) rats accumulate *C. difficile* and thus, can serve as source of infection for humans; (3) rats seem to acquire *C. difficile* due to widespread environmental contamination, and (4) rats seem to acquire *C. difficile* via indirect contact from humans or other animal species.

2.4.4.4. Environmental contamination in animal sites

Not only the isolation of *C. difficile* in faecal samples from a vast variety of species but also the evidence of environmental contamination suggests a ubiquitous distribution of the pathogen and impedes finding the source of infection. So, Weese et al. (2000a) described widespread contamination with *C. difficile* in the environment of a veterinary teaching hospital, supporting the suggestion that nosocomial CDI is also likely to occur in animal clinics. Also, soil and water samples proved to be partially highly contaminated with *C. difficile,* leading to the assumption that either individuals can become infected from environmental sources or animals shedding the pathogen contaminate the environment (Sisk et al. 1982; AlSaif and Brazier 1996; Simango and Mwakurudza 2008). Simango and Mwakurudza (2008) emphasized that endospores facilitate *C. difficile* survival over long periods which might be an underestimated fact in case of environmental contamination of soil.

On a pig farm belonging to the faculty of veterinary medicine in Utrecht, in the Netherlands, vermin were identified as vectors for *C. difficile* (Burt et al. 2012). Among the species harbouring *C. difficile* were house mice (*Mus musculus*), drain flies (*Psychoda alternate*), houseflies (*Fannia canicularis*), fruit flies (*Drosophilidae*), mealworm beetles (*Tenebrio*

molitor), house sparrows (*Passer domesticus*), and wild birds. Samples were droppings or grounded body parts (e.g. snout, gut, muscle tissue). 66% (35/53) of the mouse samples were positive for *C. difficile*, and even up to 100% (11/11) of the samples originating from insects (mealworm beetles) were positive. The predominant strain in all species was RT 078, the RT, which is mainly associated with livestock and CA-CDI in humans (Goorhuis et al. 2008b; Debast et al. 2009). Burt and her colleagues discussed that vermin could acquire the pathogen either directly from pigs or the contaminated environment via porcine faeces or dust. Keessen et al. (2011a) had earlier reported on isolating *C. difficile* from dust on pig farms. Thus, Burt et al. pointed out that trapped vermin could also function as an indicator for the carriage rates of *C. difficile* in livestock.

2.5. Food as a possible source of infection

Reports on patients suffering from PMC or CDI without prior antibiotic treatment led to the differentiation between HA-CDI and CA-CDI. Typical risk factors associated with HA-CDI are considered to be antibiosis, older age, comorbidity, and hospitalization. Cases of CDI not associated with those formerly identified and widely acknowledged risk factors led to the assumption that ingestion of contaminated food might also cause CDI (Rupnik et al. 2009b).

The first clinical case of a woman who sickened after the consumption of canned and presumably *C. difficile* contaminated salmon was published in 1982 (Gurian et al.). However, the authors were not able to examine the salmon and prove contamination with *C. difficile*. Nevertheless, Borriello et al. (1983a) stated that food-borne transmission of *C. difficile* was of obvious interest considering the fact that *C. difficile* had previously been isolated from various food-producing animal species (ducks, geese, cattle). This might indicate that those animals could function as infectious sources for food contamination. Following the report by Gurian et al. (1982), more than 200 food products were cultured for *C. difficile* (Oishi et al. 1983). The authors sampled food from the canteen of a hospital in California, USA, where CDI was commonly diagnosed. Due to the fact that all food samples were negative for *C. difficile*, Oishi et al. assumed that transmission of the bacterium from food sources is unlikely to occur.

To investigate the genetic relatedness of 97 *C. difficile* isolates originating from food, livestock and humans, Marsh et al. (2011) performed PFGE and MLVA. The 45 human isolates originated from the USA, Canada, Spain and Italy, whereas 17 of those isolates derived from CA-CDI cases, the remaining isolates were related to unclassified cases. The 26 animal isolates were obtained from porcine (n = 11), bovine (n = 14) and equine (n = 1) samples. Another 26 *C. difficile* isolates from different food samples originated from retail meat (pork, beef and turkey) from Canada and the USA. All isolates were either assigned to RT 078 (n = 60) or 027 (n = 37). Interestingly, the genetic relatedness within both RTs showed two different patterns. All isolates belonging to RT 078 were genetically closely related, regardless whether the isolates originated from food, animal or human samples, or whether the sample derived from CA-CDI or unclassified cases. In contrast, a high allelic diversity was found within RT 027, with three clusters being predominant. The isolates within the three clusters did not originate from one source but from meat products, animal and/or human samples. Moreover, they were highly related and geographically linked.

2.5.1. *C. difficile* in food products of animal origin

Rodriguez-Palacios et al. (2007) reported, for the first time, on the isolation of *C. difficile* in retail ground meat which was intended for human consumption. The meat originated from beef and veal purchased in grocery stores in Canada. 20% (12) of the 60 samples were contaminated with *C. difficile*. Eleven of the isolates were toxigenic and the comparison of meat with bovine, human and canine RTs showed no distinct difference. Based on the fact

that endospores survive the recommended cooking temperature of ground beef at 71°C even for a period of 120 min, ingestion and subsequent infection of humans, resp. cross-transmission, does not seem unlikely (Rodriguez-Palacios et al. 2007). Furthermore, it has been discussed that not only faeces but also soft tissue can harbour *C. difficile* endospores (Rupnik 2007; Vengust et al. 2003).

C. difficile could not be isolated from hamburger samples (Von Abercron et al. 2009). Curry et al. (2012) investigated uncooked porcine ground meat samples from different suppliers over a period of five months rigorously avoiding cross-contamination during laboratory processes. Although the authors applied sensitive methods for *C. difficile* detection, the overall isolation rate of 2% (2/102) was comparably low. All 82 isolates were assigned to RT 078 and all *C. difficile*-positive samples originated from the same retail meat processing facility while the other facilities tested negative. The authors discussed a probable contamination before or during processing.

Thus, contamination with *C. difficile* spores or vegetative cells implies a threat for the food producing industry. Considering the high prevalence of *C. difficile* in faeces from laying hens,

contamination of meat via faeces during food processing and the subsequent distribution into the consumers' households is likely to occur (Rupnik 2007). Also, Rupnik pointed out that this event chain raises the possibility of interspecies transmission. Countries with poorer hygienic conditions where food store chains of different food products are less fairly separated, contamination of fruit and vegetable due to *C. difficile*-positive chicken faeces might play an important role, e.g. as described in Zimbabwe by Simango and Mwakurudza (2008).

However, the prevalence of meat contamination with *C. difficile* differed significantly. In retail beef products the isolation rates ranged from 0% in the Netherlands (de Boer et al. 2011) and Austria (Indra et al. 2009), 1.9% in France (Bouttier et al. 2010), 2.4% in Sweden (Von Abercron et al. 2009), and 6.7% to 20.8 % in Canada (Rodriguez-Palacios et al. 2009; Weese et al. 2009; Rodriguez-Palacios et al. 2007). With half of the samples (50%) (13/26) being positive for *C. difficile*, Songer et al. (2009) reported the highest isolation rate in meat products of bovine origin up to now. In ground veal products isolation rates varied between 4.6% (Rodriguez-Palacios et al. 2009), to 8.0% (Houser et al. 2012) and 14.3% (Rodriguez-Palacios et al. 2007).

In porcine meat products the isolation rates for *C. difficile* ranged from 0% (0/63) in the Netherlands (de Boer et al. 2011), France (0/59) (Bouttier et al. 2010), Austria (0/27) (Indra et al. 2009) and Sweden (Von Abercron et al. 2009), 1.4% (2/148) in Germany (Maurischat et al. 2018) and 1.8% (7/393) to 12.0% (14/115) in Canada (Metcalf et al. 2010a; Weese et al. 2009). The highest isolation rates for retail meat of porcine origin was reported from the USA with 42.9% (3/7) positive samples from ground pork and even 62.5% (10/16) from ready-to-eat sausages ("braunschweiger") (Songer et al. 2009); nonetheless, due to limited sample sizes those results are not representative.

In meat products originating from poultry the isolation rates ranged from 0% in Sweden (Von Abercron et al. 2009) and Austria (0/6) (Indra et al. 2009), 2.7% (7/257) in the Netherlands (de Boer et al. 2011), 12.5% (4/32) in the US (Harvey et al. 2011), 12.8% (26/203) in Canada (Weese et al. 2010b) and 13.3% (36/270) in Germany (Maurischat et al. 2018).

Also, seafood might be a potential source of *C. difficile*, not only for humans but also for domestic animals. In Italy isolation rates in marine edible bivalve molluscs ranged from 3.9%

(36/925) (Troiano et al. 2015) up to 49.1% (26/53) (Pasquale et al. 2012). Interestingly, the isolated toxigenic RTs were similar to those from Italian patients diagnosed with HA-CDI or CA-CDI. The presence of *C. difficile* has also been proven in retail seafood and fish in North-America (Norman et al. 2014; Metcalf et al. 2011), where RT 078 was the most prevalent RT (Metcalf et al. 2011).

Despite the varying isolation rates in food products of animal origin, especially in meat products, *C. difficile* could not be detected in raw milk, so far (Jöbstl et al. 2010).

2.5.2. *C. difficile* in vegetables

Besides the isolation of *C. difficile* in food products of animal origin, it has also been detected in vegetables. In 2009, Bakri et al. (2009) reported that 7.5% (3/40) of ready-to-eat salads were contaminated with toxigenic *C. difficile* in Scotland. The authors assumed that raw consumption could lead to colonization and a subsequent increased asymptomatic carriage rate in humans. Environmental contamination or transmission by food traders were discussed as possible reasons. A convenience sample of 111 vegetables obtained from grocery stores was investigated in Canada. *C. difficile* was found in ginger, a carrot and eddoes (an Asian root vegetable); the overall isolation rate was 4.5% (5/111) (Metcalf et al. 2010b). Alarmingly, the authors detected the highly virulent RT 078 in two of the samples. The authors discussed manure fertilizer, soil and human contact during food processing as potential sources. In Germany, 401 non-animal food samples (salads and sweet peppers originating from Southern Europe or Germany) were examined throughout the winter and summer period for the presence of *C. difficile* (Maurischat et al. 2018). The authors reported an overall isolation rate of 2.0% (8/401) with detection predominance during winter. Interestingly, all isolates originated from mixed salads with a contamination rate of 3.6% (8/224). Maurischat and colleagues detected mainly toxigenic RTs (62.5%; 5/8) amongst others also well known human RTs such as RT 014/0 or 010.

Recently, *C. difficile* was detected in 28.5% (69/242) potato samples collected in 15 different countries throughout Europe; two thirds of the isolated RTs were toxigenic (Tkalec et al. 2018). Of the determined RTs, 70% (42/60) had previously been reported in samples origniating from animals, humans or the environment. The highest isolation rates of *C. difficile* in vegetables were reported by Lim et al. (2018) who sampled retail root vegetables in Western Australia. The authors detected an overall *C. difficile* isolation rate of 30.0% (30/100) with 55.6% (15/27) in organic potatoes, 50.0% (9/18) in nonorganic potatoes, 22.2% (4/18) in organic beetroots, 5.6% (1/18) in organic onions, and 5.3% (1/19) in organic carrots. The assumption that manure fertilizer might play a crucial role was supported by the high overlap in RTs isolated from root vegetables and livestock in Australia. Moreover, the authors pointed out that those RTs had also been detected in Australian patients suffering from CDI.

2.5.3. *C. difficile* in animal diets

Apart from food produced for human consumption, a variety of enteropathogens had been isolated from commercial raw diets for animals produced in the USA (Weese et al. 2005). The authors showed that not only *Salmonella* spp. but also *Clostridium perfringens* and *C. difficile* could be found in raw canine and feline diets. The meat originated from chicken and other avian species, different ruminants, rabbit, and salmon. Only the turkey-based diet was tested positive for *C. difficile.* Hence, Weese et al. advised against feeding raw canine and feline diets if young children, elderly or immunocompromised people live in the same household. Similar to food, the amount of contamination resp. detection of *C. difficile* in feed products from animal origin seemed to vary significantly in different countries, e.g. Bouttier et al. (2010) were not able to isolate *C. difficile* in feline raw diet meat in France. Accordingly,

Indra et al. (2009) indicated that European animal feed products seem to be less often contaminated with *C. difficile*.

3. Material

3.1. Project pseudonym and symbol

For advertisement and acquisition purposes a project pseudonym and symbol (Figure 2) with considerable recognition value was created. The project pseudonym was intended to display the main aim of the study with the central question: Is human CDI associated with small companion animals harbouring potentially zoonotic *C. difficile*? Thus, the pseudonym had to comprise the abbreviations of *C. difficile*, infection and zoonosis, resulting in the pseudonym "CDi.zoo". The pseudonym was then integrated into the project symbol illustrating the three study groups of humans, dogs and cats.

Figure 2: Project pseudonym and symbol

3.2. Data collection

To identify factors significantly associated with shedding of *C. difficile* in small animals and their owners a questionnaire was developed comprising relevant questions derived from a comprehensive literature review. To ensure an unambiguous visual assignment all material necessary for data and sample collection were colour coded; thereby, identification labels, numbers and the "animal part" of the questionnaire was coloured in purple resp. in green for participating animal owners.

3.2.1. Identification numbers

To secure data privacy every single study participant was assigned to an identification number (ID); consequently all data belonging to one participant were encoded with a tag assigned to the personal ID. The procedure of ID assignment, data processing and information management was approved by the data protection working group belonging to the TMF (Technologie- und Methodenplattform für die vernetzte medizinische Forschung e.V., Technology, Methods, and Infrastructure for Networked Medical Research). The TMF is intended as an umbrella organization for networked medical research in Germany (TMF 2017). The approval and positive evaluation of the study outline was based on a presentation on the 11th of September 2012 during a group meeting.

In addition to the random assignment of ID numbers, encoding the participating household, a letter enciphering the study group, either "T" for animal ("Tier") or "M" for human ("Mensch"), and a number encoding the number of participating animal or human within the household were affixed to all corresponding data sheets; e.g. "0681M1".

3.2.2. Questionnaires

The questionnaire (see original questionnaire within the Appendix) was designed in cooperation with the Robert Koch Institute (RKI), the federal central scientific institution for biomedicine, and the Friedrich-Loeffler-Institute, the Federal Research Institute for Animal Health. The questionnaire comprised of 45 questions and was separated into four main topics: (1) General information about animal contacts, (2) Information about the tested animal, (3) Information about the animal owner, and (4) Miscellaneous.

3.2.2.1. ID numbers

Every single questionnaire was tagged with the corresponding ID. The first double printed page aiming at the animal participant was tagged with the animal ID, e.g. “0283T2” encoding for the second participating animal of the household with the ID 0283. The second double printed page targeted the animal owner and was consequently tagged with the corresponding ID, e.g. “0283M2” encoding for the second participating human of the household with the ID 0283.

3.2.2.2. Part 1 – General information about animal contacts

The first part of the questionnaire aimed at requesting information about further animal contacts of the animal owner apart from the dog and/or cat participating within the study (Figure 3). This part was coloured in blue to distinguish it easily from the remaining purple and green coloured parts of the questionnaire.

Figure 3: Part 1 of the questionnaire requesting general information about animal contacts

I. General information about animal contacts

1. Have you got other pets or animals apart from the participating animal?
☐ Yes ☐ No ☐ n/a

↳ **if applicable, which animals? (check all that apply) – Please, specify the quantity**.....

.....**dogs** .....**cats** .....**sheep** .....**poultry** .....**wild animals** .....**rabbits and other small mammals**

.....**horses** .....**cattle** .....**pigs** .....**others (please specify):** .....

2. Have you been several times in contact with other animals (of which you are not the owner) during the last 12 months? (e.g. pets, hunt…)?
☐ Yes ☐ No ☐ n/a

↳ ***if applicable,* which animals? (check all that apply)**
☐ dogs ☐ cats ☐ sheep ☐ poultry ☐ wild animals ☐ rabbits and other small mammals
☐ horses ☐ cattle ☐ pigs ☐ others (please specify): .....

3.2.2.3. Part 2 – Information about the tested animal

The second part of the questionnaire was designed to gain information about the participating animal and included a field for tagging (Figure 4). This part of the questionnaire was framed in purple.

Figure 4: Field for the animal ID belonging to part 2 of the questionnaire

Part 2 comprised of 22 questions (question 3 to 23) (Figure 5) requesting information about demographic factors such as species, breed, age, sex, and whether the animal was neutered as well as details of husbandry (keeping inside/free roaming), stay in different sites (e.g. sanctuary, animal shows), contact between pet and human, feed consumption, status of health prior hospitalization, intake of medication (e.g. antibiotics), and contact to other individuals suffering from diarrhoea or with a recent hospital stay.

Figure 5: Part 2 of the questionnaire requesting information about the animal participant

II. Information about the tested animal

3. The tested animal is a: ☐ **dog** ☐ **cat**

4. Which species does the animal belong to?	**5. Gender?** ☐ **female** ☐ **male**	**6. Neutered?** ☐ **Yes** ☐ **No**	**7. Age:** ..... **years**
.....			

8a) ***(if tested animal is a dog)*** **Where is the dog most of the times (more than half of the time)?**
☐ **in the house/flat**
☐ **in a kennel/garden**
☐ **in a separate building (e.g. in a stable)**

8b) ***(if tested animal is a cat)*** **Where is the cat most of the times?**
☐ **in the house/flat**
☐ **outdoors**

↳ ***if the tested animal is an outdoor-cat,*** **how often is it outside?**
☐ **daily:** ..... **hours per day**
☐ **several times a week:** ..... **hours per week**

9. Is the animal in regular contact with other companion animals or livestock?
☐ **Yes** ☐ **No** ☐ **n/A**

↳ ***if applicable,*** **which animals? (check all that apply)**
☐ **dogs** ☐ **cats** ☐ **sheep** ☐ **poultry** ☐ **wild animals** ☐ **small mammals**
☐ **horses** ☐ **cattle** ☐ **pigs** ☐ **others (please specify):** .....

10. Has the tested animal been in contact with neonates/young animals during the last 12 months (e.g., dog puppies, piglets, …)?
☐ **Yes** ☐ **No** ☐ **n/A**

↳ ***if applicable,*** **which animals? (check all that apply)**
☐ **dogs** ☐ **cats** ☐ **sheep** ☐ **poultry** ☐ **wild animals** ☐ **small mammals**
☐ **horses** ☐ **cattle** ☐ **pigs** ☐ **others (please specify):** .....

11. Which of the following is applicable describing the contact between owner and animal during the last 12 months?

The animal is …	**Daily**	**Several times per week**	**Several times per month**	**Rarely**	**Never**
a) …allowed to lie on the couch	☐	☐	☐	☐	☐
b) …allowed to sleep in the owner's bed	☐	☐	☐	☐	☐
c) …washed in the tub/shower	☐	☐	☐	☐	☐
d) …petted	☐	☐	☐	☐	☐
e) …allowed to feed out of the hand	☐	☐	☐	☐	☐
f) …is allowed to lick the owner's face	☐	☐	☐	☐	☐
g) other contacts	☐	☐	☐	☐	☐

(please specify):.....

12. Has the tested animal been in any of the following facilities during the last 12 months?

a) kennels/boarding facilities	☐ **Yes: when at last (month/year)?** ...../.....	☐ **No**
b) animal shelter	☐ **Yes: when at last (month/year)?** ...../.....	☐ **No**
c) kindergarten, school	☐ **Yes: when at last (month/year)?** ...../.....	☐ **No**
d) dog-/cat-show	☐ **Yes: when at last (month/year)?** ...../.....	☐ **No**
e) health care/rehabilitation facility	☐ **Yes: when at last (month/year)?** ...../.....	☐ **No**
f) in use as a therapy dog (e.g. in hospital)	☐ **Yes: when at last (month/year)?** ...../.....	☐ **No**
g) pet obedience school	☐ **Yes: when at last (month/year)?** ...../.....	☐ **No**

(Continued on following page)

...more information regarding the tested animal

13. Which of the following does the tested animal feed on? (check all that apply)

- ☐ canned feed
- ☐ dry feed
- ☐ jerky (e.g. dried pig ears)
- ☐ raw meat (products) (e.g. rumen)
- ☐ leftovers
- ☐ dog/cat treats (e.g. dog chew bones, cat chew treats)
- ☐ nutritional supplements (e.g. vitamin or mineral additives)
- ☐ others (please specify): .....

14. Is the tested animal prone to …?

a) feeding on faeces (coprophagy)	☐ Yes	☐ No	☐ unknown
b) uncontrolled greedy feeding, e.g. consumption of waste (polyphagia)	☐ Yes	☐ No	☐ unknown
c) loss of appetite (inappetence)	☐ Yes	☐ No	☐ unknown

15. Does the tested animal suffer from an acute disease? (short period of 3-14 days)

☐ Yes, from: ..... ☐ No ☐ unknown

16. Does the tested animal suffer from a chronic disease? (long period over 14 days, e.g. diabetes, skin disease, tumor)

☐ Yes, from: ..... ☐ No ☐ unknown

17. Does the tested animal regularly take in anti-inflammatory drugs (e.g. Metacam®, Rimadyl®)?

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable*, since when? (month/year)? ..../.....

18. Does the tested animal regularly take in drugs to reduce gastric acidity (so called proton pump inhibitors, e.g. Omeprazol)?

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable*, since when? (month/year)? ..../.....

19. Has the tested animal been treated with antibiotics during the last 3 months? (no anthelmintic therapy)

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable:* Why? Diagnosis/Disease: .....
When? (month/year)? ..../.....
How? ☐ local treatment (ointment, eyedrops)
☐ systemic treatment (drugs, syringe)

20. Has the tested animal suffered from diarrhoea during the last 4 weeks? (diarrhoea as in terms of more than 3 unformed faeces/day)

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable*, how long did the diarrhoea last?

☐ a few days until max. 3 weeks ☐ longer than 3 weeks ☐ n/A

21. Has the tested animal been in contact with a human or animal suffering from diarrhoea?

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable:* ☐ to a human. When at last? (month/year)? ..../.....
☐ to an animal. When at last? (month/year)? ..../.....

22. Has the tested animal been hospitalized in a veterinary clinic during the last 12 months?

☐ Yes: When at last? (month/year)? ..../..... ☐ No ☐ unknown

23. During the last 12 months has the tested animal been in contact with a patient (human or animal) who had been recently hospitalized?

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable:* ☐ to a human. When at last? (month/year)? ..../.....
☐ to an animal. When at last? (month/year)? ..../.....

3.2.2.4. Part 3 – Information about the animal owner

The third part of the questionnaire was designed to gain information about the participating animal owner and also included a field for tagging (Figure 6). This part of the questionnaire was framed in green.

Figure 6: Field for the animal owner ID belonging to part 3 of the questionnaire

Part 3 comprised of 20 questions (question 24 to 43) requested demographic data like age, gender, profession, residence and residential environment (e.g. city, countryside). Additional questions assessed the presence of other household members such as children and individuals with chronic disease, confirmed CDI or persons who underwent chemotherapy. Moreover, additional information was collected about the intensity of contact between pet and owner (e.g. frequency and type of dog handling, including physical contact), food consumption, status of health, prior hospitalization, intake of certain pharmaceuticals, and contact to other individuals suffering from diarrhoea or with a recent hospital stay (Figure 7).

Figure 7: Part 3 of the questionnaire requesting information about the animal owner

III. Information about the animal owner

24. In which district of Germany do you live? .....

25. How would you describe your residential environment?
- ☐ large city
- ☐ provincial city
- ☐ countryside
- ☐ n/A

26. Date of birth (month/year)? ...../.....

27. Gender

☐ female ☐ male

28. Which is your current field of occupation (check all that apply)?
- ☐ in agriculture, as: .....
- ☐ in food production, as: .....
- ☐ in health care, as: .....
- ☐ other field of occupation: .....
- ☐ currently not occupied (parental leave, retirement, etc.)
- ☐ n/A

29. Do children younger than 16 years of age live in your household?

☐ Yes ☐ No ☐ unknown

↳ *if applicable,* please specify how many children in the following age groups:

number of children younger than 2 years: ..... child/ren

number of children between 2 and 9 years: ..... child/ren

number of children between 10 and 16 years: ..... child/ren

30. Does a chronically sick person live in your household?

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable,* which disease? .....

31. Has a person or animal living in your household previously been tested positive for *C. difficile*?

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable:* ☐ a person. When? (month/year)?...../.....

☐ an animal. When? (month/year)?...../.....

32. Which of the following food/drinks do you consume?

	Daily	Several times per week	Several times per month	Rarely	Never
tap water as a cold drink	☐	☐	☐	☐	☐
raw milk/-products	☐	☐	☐	☐	☐
raw meat/-products (e.g. mince)	☐	☐	☐	☐	☐
ready-to-eat-salads	☐	☐	☐	☐	☐
probiotics (e.g. Actimel®)	☐	☐	☐	☐	☐

33. Have you been in contact with a human or animal suffering from diarrhoea during the last 12 months?

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable:* ☐ with a human. When at last? (month/year)?...../.....

☐ with an animal. When at last? (month/year)?...../.....

34. Have you been hospitalized for at least one week during the last 12 months?

☐ Yes: When was the last stay? (month/year)?: ...../..... ☐ No ☐ unknown ☐ n/A

(Continued on following page)

… more questions regarding the animal owner

35. During the last 12 months have you been in contact with a patient (human or animal) who had been recently hospitalized?

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable:* ☐ with a human. When at last? (month/year)? ..../.....

☐ with an animal. When at last? (month/year)? ..../.....

36. Have you suffered from diarrhoea during the last 4 weeks?
(diarrhoea as in terms of more than 3 unformed faeces/day)

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable*, how long did the diarrhoea last?

☐ a few days until max. 3 weeks ☐ longer than 3 weeks ☐ n/A

37. Do you regularly take in anti-inflammatory drugs (e.g. Aspirin®, Ibuprofen)?

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable*, since when? (month/year)? ..../.....

38. Do you regularly take in drugs to reduce gastric acidity?
(so called proton pump inhibitors (e.g. Nexium®, Pantozol®)

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable*, since when? (month/year)? ..../.....

39. Have you been in contact to a diarrhoeic patient (human or animal) during the last 12 months?

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable:* ☐ to a human. When at last? (month/year)? ..../.....

☐ to an animal. When at last? (month/year)? ..../.....

40. Have you been treated with antibiotics during the last 2 months?

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable:* Why? Diagnosis/Disease: .....
When? (month/year)? ..../.....

How? ☐ local treatment (ointment, eyedrops)

☐ systemic treatment (drugs, syringe)

41. Have you received chemotherapy during the last year?

☐ Yes ☐ No ☐ n/A

42. Do you suffer from a chronic disease (e.g. diabetes, neurodermitis, among others)?

☐ Yes ☐ No ☐ n/A

↳ *if applicable,* which disease? .....

43. Have you previously been positively tested for *C. difficile*?

☐ Yes ☐ No ☐ unknown ☐ n/A

↳ *if applicable*, did you suffer from associated symptoms?

☐ Yes, such as: ..... ☐ No ☐ unknown ☐ n/A

3.2.2.5. Part 4 – Miscellaneous

The last part of the questionnaire was separated from the previous three parts as it did not request clinical or epidemiological data but included an administrative question concerning further contacts to the study participant, number 44 (Figure 8).

Figure 8: Part 4 of the questionnaire requesting contact allowance from the animal owner

IV. Miscellaneous

44. Do you allow us contacting you in case additional information is necessary within the survey?
☐ **Yes** ☐ **No**

3.2.3. Software for data entry and analysis

3.2.3.1. EpiData

The software EpiData is a Windows 95/98/NT/2000 based program (32 bit) and is available as freeware from http://www.epidata.dk (Lauritsen and Bruus 2003-2008) due to voluntary work and funding (EpiData). EpiData consists of applications for data entry and the subsequent analysis of quantitative data. EpiData is widely used, e.g. the World Health Organization (WHO), for editing large data sets (Wikipedia). EpiData enables complete export of the generated data sets for further enhanced analysis in various data file formats into other programs, such as STATA. EpiData, version 3.1., was used to document and digitalize the completed questionnaires.

3.2.3.2. Stata®

Statistical analysis was performed using the software Stata® (StataCorp. 2013. Release 13. College Station, TX: StataCorp LP). Data sets created in EpiData were exported as complementary data files (.qes, .chk, and .rec files) and the subsequently created .dta file was imported into Stata. To ensure objective traceability and confirmability Do-Files (.do files) were produced (Kohler and Kreuter 2012). Those Do-Files enabled the interdisciplinary communication between project partners during the process of the analysis of univariate and multivariate variables associated with faecal shedding of *C. difficile* in animal and human participants.

3.4. Sample collection

To ensure successful self-sampling each sampling kit was individually packed and delivered either directly or via post. It included individually labeled stool containers for self-sampling, information and instruction sheets, consent forms, as well as the self-reporting questionnaire for each participating household member (see Appendix).

3.4.1. Personal letter of instruction

A personal letter of instruction was attached to the sampling package. It included information about the content of the sampling package, the personal ID number, contact data, notification of microbiological results, and the information about the fulfilment of the data privacy laws. The participant was also informed about the necessity of completing consent forms and questionnaires and their return using the already labelled and post-paid envelope to the Institute of Microbiology and Epizootics, Freie Universität Berlin (FU Berlin), to fulfil the inclusion criteria. Moreover, the letter included information about the delivery of the faecal samples to the Institute of Bacterial Infections and Zoonoses at the Federal Research Institute for Animal Health (Friedrich-Loeffler-Institut) in Jena using the attached package.

3.4.2. Transport of biological substances

The packaging requirements for the transport of biological material are regulated by the European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR) (UNECE 2010). According to the ADR samples collected within this study are categorized in Class 6.2 as category B and assigned to UN No. 3373; thus, for shipping it has to be labelled as "BIOLOGICAL SUBSTANCE; CATEGORY B". The packaging has to comply with the requirements of a three-component-system consisting of (1) a leak-proof primary receptacle; (2) a leak-proof secondary packaging; and (3) an outer packaging of adequate strength for its capacity, mass and intended use, and with at least one surface having minimum dimensions of 100 mm x 100 mm.

3.4.2.1. Primary receptacle

The faeces tube by Sarstedt AG & Co. (Nürnbrecht) was used as it met the requirements of the ADR as a leak-proof primary receptacle for faecal samples and enabled convenient sampling due to its integrated spoon. The white faeces container measured 54 x 28 mm, was made of polypropylene and had a brown screw cap with an integrated spoon and a standard paper label (Sarstedt 2017). Additionally, the faeces tube met the requirements for convenient posting as the size matched with the secondary and outer packaging, with the latter having restricted dimensions as it had to fit into standard German post boxes.

Each faeces tube was labelled with a sticker showing a pictogram of the corresponding study group (either human or dog and cat) and the ID number coloured in green for human and in purple for animal participants. Those precautions aimed to ensure correct assignment of the faeces tubes to the corresponding participant resp. participating household member.

3.4.2.2. Secondary packaging

The patented Safetybag (DaklaPack®, Leylstad, NL) was used as a secondary leak-proof packaging. The Safetybag was made of polyethylene and tested as suitable as a secondary liquid tight packaging for transport of biological substances of category B. The bag measured 165 x 170 mm. The signs "P650", "UN3373" within a rhomboid symbol, and "Biological Substance, Category B" were printed on the front of the Safetybag's outer surface.

3.4.2.3. Outer packaging

The only packaging meeting the regulatory formalities of the ADR and our requirements of a packaging height less than 35 mm to fit into a standard German post box was the patented MiniMailBox (DaklaPack®, Leylstad, NL). The cardboard box had a white outer surface labelled with the signs "P650", "UN3373" within a rhomboid symbol, and "BIOLOGICAL SUBSTANCE, CATEGORY B" and measured 240 x 129 x 30 mm (DaklaPack 2017).

The MiniMailBox was equipped with all the components necessary for a convenient and successful participation for all interested household members (humans and animals): (1) instruction sheet for sample handling and posting; (2) information sheet about the study design; (3) consent forms; (4) questionnaires; (5) prepared return envelope; (6) disposable gloves; (7) stool specimen collector; and (8) faeces tubes. The outer packaging was tagged with a posting sticker enabling the postage payment at arrival at the recipient's address.

3.4.3. Instruction sheet for sample handling and posting

Each sampling kit included an instruction sheet which was divided into four parts: (1) content of the sampling kit, (2) sample collection for animal owners, (3) sample collection for animal participants, and (4) posting the samples. Each description was additionally illustrated with photos or pictograms. Furthermore, participants were encouraged to use disposable gloves for safe and hygienic sample collection. Coloured labels and pictograms of the study groups

(human or dog and cat) on the faeces tubes additionally ensured the correct assignment of faeces tubes to the corresponding household participants.

3.4.4. Information sheet about study design

Supplementary to participant information via direct contact, each sampling kit included an information sheet about the study design covering the characteristics of the bacterium *C. difficile* and its role as an important pathogen, the major aims of the study, a description of the participating institutes and their tasks within the scope of the study, fulfilment of the data privacy laws and the notification of microbiological results. Finally, complete contact data were provided for further enquiry.

3.4.5. Declaration of consent

The forms of declaration of consent for human and animal participants were designed based on the legal recommendations by Goebel and Scheller (2012).

3.4.5.1. Declaration of consent for human participants

Each participating human household member received a duplicated double-printed form to declare with signature and date of signature the right of rescission and acceptance of the participation conditions. The declaration form was labelled with the corresponding ID number in green. Moreover, the participant had to decide whether his/her faecal sample can be used for other studies. Finally, the responsible staff members acknowledged with signature and date of signature the oral and written study participant information. The original declaration(s) of consent of every household participant had to be returned to the Institute of Microbiology and Epizootics, FU Berlin, for assessment and archiving.

3.4.5.2. Declaration of voluntary surrender for animal samples

Each participating household received a duplicated form to declare with signature and date of signature that the faecal sample(s) of the participating animal(s) was/were surrendered to the research project and its participating institutes. The declaration form was labelled with the corresponding ID number(s) in purple for all participating animals within the household. The original declaration of surrender had to be returned to the Institute of Microbiology and Epizootics, FU Berlin, for assessment and archiving.

3.4.6. Questionnaires

Questionnaires for each participating household member were also attached to the content of the sampling kit. The questionnaire comprised of 44 questions and was divided into: (1) General information about animal contacts, (2) Information about the tested animal, (3) Information about the animal owner, and (4) Miscellaneous (Further contacts) (see 3.2.2).

3.4.7. Disposable gloves

Each sampling kit also included two disposable gloves for each human participant within one household. The gloves rotiprotect-Vinyl (Carl Roth GmbH & Co., Karlsruhe) were transparent, non sterile, ambidextrous, made of synthetical vinyl-polymer, free from natural rubber latex and sustainable for CE Category I (CarlRoth 2017).

3.4.8. Stool specimen collector

Each sampling kit additionally included a stool specimen collector (Süsse Stuhlfänger, MED AUXIL, Bad Gandersheim) for each human participant for safe and hygienic faecal collection.

[*Author's comment: Material for the bacterial isolation and characterization will not be described here as this was the main task of the project partner the Friedrich-Loeffler-Institute. However, methods of the microbiological sample examination will be described briefly in the following chapter as the microbiological results formed the basis for the statistical analysis of the collected data. For additional information see: Schneeberg et al. (2015) and Schneeberg (2016).*]

4. Methods

4.1. Partners involved in the interdisciplinary project

The pilot project "Survey on prevalence and molecular characterization of *Clostridium difficile* from small companion animals and their owners" was funded by the German Federal Ministry of Education and Research (BMBF) (funding number: "Clostridium difficile" 01KI1107/01KI1108) from 1st March 2012 for 18 months until 31st August 2013 with a subsequent extension of the funding period exempt from charges until 30th April 2014. This interdisciplinary research project involved three project partners and was supported by the German Research Platform for Zoonoses.

4.1.1. Freie Universität Berlin, Institute of Microbiology and Epizootics

The project was conducted by Dr. Antina Lübke-Becker from the Institute of Microbiology and Epizootics, Department of Veterinary Medicine, FU Berlin. The main tasks of this project partner were: (1) study participant recruitment (including preparation and submission of the application for an ethical approval and the logistics of the sample collection and shipping), (2) draft of a questionnaire, (3) statistical analysis of the epidemiological data, and (4) coordination and conjunction of other work packages.

4.1.2. Friedrich-Loeffler-Institute (FLI)

The workgroup of Clostridiae led by Dr. Christian Seyboldt is part of the Institute of Bacterial Infections and Zoonoses at the Federal Research Institute for Animal Health (Friedrich-Loeffler-Institute (FLI)). The main tasks of the FLI under the scope of this project were: (1) isolation of *C. difficile* from faecal samples of dogs, cats and animal owners, (2) molecular characterization of the isolated strains, and (3) establishment of a biomaterial bank for all collected and approved faecal samples.

4.1.3. Robert Koch Institute (RKI)

The main tasks of the Department of Infectious Disease Epidemiology, FG 32 Surveillance, Robert Koch Institute (RKI), led by Dr. Tim Eckmanns were: (1) supporting the application for the ethics approval of the study design (including finalising of essential documents drafted by the FU Berlin such as the instruction sheet for sample handling and posting, the information sheet about study design, the consent forms, the questionnaires), (2) essentially supporting the statistical analysis of the epidemiological data, (3) providing data from a previously conducted survey called "Mick study" with human-specific data for comparison, and (4) being the contact point for study participants for all questions which needed to be answered by medical physicians.

4.2. Ethics approval

The application for an ethics approval had been submitted to the Ethical Committee of the Charité, Campus Virchow-Clinic Berlin, on the 21st of May, 2012. The request comprised the following documents: (1) flyer, (2) instruction sheet for sample handling and posting, (3) information sheet about study design, (4) declaration of consent for human participants, (5) declaration of surrender for animal samples, and (6) questionnaires. The request (application number: EA2/070/12) was discussed in detail during the meeting of the ethical committee on the 7th of June, 2012. Finally, the study protocol was approved by the Ethical Committee of the Charité Berlin on the 14th of June, 2012.

4.2.1. Data privacy

To ensure that name and address of the participants was only traceable by one responsible person, consent forms, questionnaires and samples had to be handled separately and were only

processed using the corresponding ID numbers. For this reason participants sent their ID number labelled samples to the FLI without any reference to the senders. The filled in consent forms and questionnaires were also labelled with the corresponding ID numbers and sent to Denise Rabold, the responsible person who was able to trace back ID and the participants' contact data for notification of the results of the microbiological examination.

4.3. Data acquisition

Definition of inclusion criteria, study participant recruitment as well as appropriate data processing and statistical analysis are essential prerequisites for epidemiological studies.

4.3.1. Study participant recruitment

According to the study design sampling of 500 individuals per study group (animal owners, dogs and cats) was intended. Study participant recruitment was accomplished between the 4th of July, 2012, and the 19th of August 2013.

4.3.1.1. Flyer

For study participant recruitment and to make the project known to interested animal owners a flyer was developed (see Appendix). The flyers were distributed among pet-owning acquaintances, family members, and colleagues, participants on conferences, general practitioners, veterinarians and pet owners attending the Small Animal Clinic (FU Berlin). Additionally, all participants received a flyer for redistribution among further interested animal owners.

4.3.1.2. Web pages

To make the survey known to a wider public and to acquire study participants, the official web page of the Institute of Microbiology and Epizootics at the FU Berlin presented the pilot project under the following URL: http://www.vetmed.fu-berlin.de/einrichtungen/institute/we07/studium/aktuelles/index.html; last available under the URL: http://www.vetmed.fu-berlin.de/einrichtungen/institute/we07/studium/aktuelles/cdi_zoo/index.html (accession date: 03/30/2017).
In addition, the German Research Platform for Zoonoses provided the opportunity to present the survey on their web page using the URL:
http://www.zoonosen.net/Projekte/Pilotprojekte/Clostridiumdifficile.aspx; currently available under the URL: http://www.zoonosen.net/Zoonosenforschung/ProjektederZoonosenplattform/AbgeschlosseneProjekte/Clostridiumdifficile.aspx (accession date: 03/30/2017).

4.3.1.3. Mailing lists, internet forums, social networking service and newsletters

Mailing lists of veterinary students in Berlin and Hannover and of the university medical centre in Münster were deployed for further participant recruitment. Additionally, information about the study outline, the inclusion criteria and a call for participation were posted on several internet forums created for veterinarians or animal owners, e.g. Foren4Vet (Foren4Vet 2013) or Felidae Katzenforum (Felidae-Katzenforum 2017). Moreover, an account was created on the social networking service facebook to build bonds with animal owners in Germany and advertise the pilot project.

In December 2012, the pilot project was advertised in the newsletters by Vetion.de GmbH which is an internet forum for animal health and veterinarians (Vetion.de 2017), "Der Praktische Tierarzt" and "Kleintierpraxis" (schlütersche 2017) both are veterinary professional journals by Schlütersche Verlagsgesellschaft mbH & Co. KG.

4.3.1.4. Cooperation with veterinary clinics, general and veterinary practitioners

Direct participant recruitment was conducted in the Small Animal Clinic at the FU Berlin.

Another veterinary clinic specialized on gastroenterology in Cologne, Germany, supported the participant recruitment. Additionally, one general practitioner located in Bochum, Germany, specialized on internal medicine, and five veterinary practitioners located in Berlin and Sassnitz, Germany, were involved in advertising the study. The practitioners were provided with flyers and a poster to call attention; they were instructed to inform clients who were potential participants about the study design and criteria for participation. Interested animal owners were then given a prepared sampling kit for home-side self-sampling and shipping of the collected samples and filled in forms.

4.3.1.5. Cooperation with the German National Cohort

As part of the German National Cohort (GNC), a large-scale population-based study investigating the causes of underlying major chronic diseases, such as diabetes or cancer with a planned duration of 25-30 years, two series of pre-tests were conducted in 2011 and 2012 to test the implementation of intended procedures (NAKO 2018). In the context of zoonoses research, feasibility tests were conducted, e.g. to investigate whether the method of animal owners sampling their own cats and/or dogs was feasible (Hille et al. 2014). The second pre-test for the GNC was carried out in 2012, and in cooperation with the Department of Biometry, Epidemiology and Information Processing, University of Veterinary Medicine Hannover, Foundation, in Hannover, Germany, and the Helmholtz Centre for Infection Research participants of the feasibility tests and their companion animals could be included in the study presented here.

4.3.1.6. Symposium, congress and international trade fair

The pilot project had also been presented to gain awareness during a poster session at the National Symposium on Zoonoses Research in Berlin (Hildebrand et al. 2012), at the industrial exhibition of the 77th annual congress for veterinary practitioners, bpt-Kongress (bpt-academy 2012), in Hannover, Germany, and at the International Green Week Food Fair in Berlin in 2013.

4.3.1.7. German dog and cat kennel clubs

German dog breeding clubs are mainly organized under the federally structured umbrella organization German Kennel Club (VDH, "Verband für das Deutsche Hundewesen"). During participant recruitment 104 kennel clubs were contacted via e-mail and asked for support through distributing the project flyer via e-mail or advertising.

In contrast to the professional organization of German dog breeding club's by the VDH, cat breeders mainly operate individually as there is no head organization comparable to the VDH. Therefore, study participant recruitment was implemented via individual e-mail contacts to private cat breeders or breeding clubs listed on web pages, e.g. "Felidae Katzenforum" (Felidae-e.V. 2017) or "Zuchtverzeichniss" (Zuchtverzeichniss 2017). In total, 22 different feline breeding clubs and 253 private cat breeders were contacted via e-mail.

4.3.2. Electronic data processing

Digitalization of the questionnaire data using EpiData software was accomplished by the cooperation partner RKI. Prior to this, a simultaneous data entry test of 50 randomly chosen questionnaires had been performed by the Institute of Microbiology and Epizootics and the RKI to avoid major errors and ensure conformity of data entry.

4.3.2.1. Sensitivity analysis

To avoid potential clustering and guarantee unbiased evaluation of epidemiological data, sensitivity analyses were performed. For this, the following four models had been defined:

(1) Only households participating with one animal and one pet owner were included (all households participating with more than one animal and one owner were excluded),

(2) All households were considered, though, only one data set (ratio animal-pet owner 1:1) was included (all other household members were excluded), the included data set was defined to consist of the animal owner with the ID number "(X)M1" and the corresponding animal with the ID number "(X)T1",

(3) All households were considered, though, only data sets with the ratio animal-pet owner n:n were included (e.g. included data sets from a household with the ratio animal-pet owner 3:2 were "(X)M1, "(X)T1" and "(X)M2, "(X)T2", and "(X)T3", the latter having been excluded),

(4) All households were included with complete data sets (ratio animal-pet owner n:m).

Since no significant advantages concerning efficiency or performance could be observed in the univariate analysis using model (1), (2) or (3), the whole data set (model (4)) was selected for the analyses.

4.3.3. Risk assessment

The statistical analyses of epidemiological factors potentially associated with faecal shedding of *C. difficile* in small companion animals and their owners in Germany were performed in cooperation with the RKI using the software STATA® (StataCorp. 2013. Release 13. College Station, TX: StataCorp LP). (The study presented here aimed to describe the *status quo* in the defined study population regarding the detection resp. faecal shedding of *C. difficile*. Within this context a differentiation between colonization, infection or transient passage is neither feasible nor relevant. To meet the considerations of all three probable statuses, the abbreviation CITP was used in accordance to the term of faecal shedding.)

4.3.3.1. Inclusion criteria

To participate in the study and be included in the assessment, at least one person (animal owner) and at least one dog or cat, living in the same household, had to meet the inclusion criteria which were residency in Germany, signed consent forms, filled in questionnaires and one faecal sample per participating household member.

4.3.3.2. Univariate analysis

Univariate analysis examined the association (odds ratio) between potential exposure and outcome concerning *C. difficile*-CITP using logistic regression with dichotomous and categorical independent variables at a significance level of $p \leq 0.05$. In total, 47 resp. 36 variables had been defined for animal resp. human participants (Table 1 and Table 2).

The variable "age" of animal participants had been categorized into four groups: (1) younger than one year, (2) between 1 and up to 4 years of age, (3) between 5 and up to 9 years, and (4) from 10 years and older. The age of human participants had been categorized into six groups of (1) younger than one year, (2) between 1 and up to 4 years of age, (3) between 5 and up to 17 years, (4) between 18 and up to 44 years, (5) between 45 and up to 64 years, and (6) from 65 years of age and older. Odds ratios (OR) were calculated with a 95% confidence interval (CI).

The variables describing the intensity of contact between animal and owner (question 11) and requesting habits of food consumption in human participants (question 32) were linked to an ordinal scale rating the frequency (daily, several times per week, several times per month, rarely, and never). To ensure an unbiased outcome, three different models were applied for

statistical analysis: (1) the variable was evaluated independently of frequency, (2) for each variable the five different frequencies were evaluated as independent, separate variables, and (3) different methods of categorisation were applied to assess the subjectively evaluated frequencies unbiasedly.

Moreover, two separate questions requested the contact between the participating animal owner and an animal or human suffering from diarrhoea (questions 33 and 39), thereby, enabling to evaluate the reliability and attentiveness of participants.

Table 1: Variables created for animal participants

Demographic factors and animal housing	
1.	Cat (dog)
2.	Breed
3.	Gender - female (male)
4.	Neutered (not neutered)
5.	Age in years
6.	Animal housing
Contact to other animals	
7.	Regular contact to other farm or companion animals
8.	Regular contact to infant animals during the last 12 months
Contact between animal and owner: The animal is allowed to…	
9.	**...** lie on the couch
10.	… sleep in bed
11.	… be washed in the tub/shower
12.	… be petted
13.	… feed out of the hand
14.	… lick the owner's face
15.	… other contacts
Stay in different sites during the last 12 months	
16.	Boarding kennel
17.	Animal shelter
18.	Kindergarten/school
19.	Dog-/cat-show
20.	Health care/ rehabilitation facility
21.	In use as therapy dog
22.	Pet obedience school
Feed consumption	
23.	Canned feed
24.	Dry feed
25.	Jerky
26.	Raw meat (products)
27.	Leftovers
28.	Dog/cat treats
29.	Animal feed additives
30.	Other feed products

Health status/ behaviour	
31.	Coprophagy
32.	Polyphagia
33.	Inappetence
34.	Acute disease
35.	Chronic disease
36.	Diarrhoea during the last 4 weeks
Medication	
37.	Anti-inflammatory drugs
38.	Proton pump inhibitors
39.	Antibiotics
Contact to health-impaired individuals/ hospitalization	
40.	Contact to a human or animal with the onset of diarrhoea during the last 12 months
41.	Owner of the tested pet has suffered from diarrhoea during the last 4 weeks
42.	Hospitalization in a veterinary clinic during the last 12 months
43.	Contact to a hospitalized human or animal during the last 12 months
44.	Person with chronic disease lives in the household
45.	Owner of the tested pet suffers from a chronic disease
46.	Person or animal with a previous positive test for *C. difficile* lives in the household
47.	Owner of the participating pet was previously tested positive for *C. difficile*

Table 2: Variables created for human participants

Demographic factors	
1.	Age in years
2.	Gender - female (male)
3.	District of Germany
4.	Place of residence (countryside/city)
5.	Profession/ field of occupation
Contact between animal and owner: The animal is allowed to…	
6.	... lie on the couch
7.	… sleep in bed
8.	… be washed in the tub/shower
9.	… be petted
10.	… feed out of the hand
11.	… lick the face
12.	… other (intense) contacts
Contact to other animals	
13.	Animal husbandry (additionally to tested dog/cat) - Keeping farm or companion animals
14.	Multiple contacts to other animals not in care by participant during the last 12 months
Contact between participating dog/cat and others	
15.	Regular contact to other farm or companion animals
16.	Regular contact to infant animals during the last 12 months
17.	Contact to a human or animal with the onset of diarrhoea during the last 12 months
Food consumption	
18.	Tap water as cold drink
19.	Raw milk/-products
20.	Raw meat/-products
21.	Ready-to-eat-salads
22.	Probiotics
Health status	
23.	Diarrhoea during the last 4 weeks
24.	Chronic disease
25.	Chemotherapy during the last 12 months
26.	Previous positive test for *C. difficile*
Medication	
27.	Anti-inflammatory drugs
28.	Proton pump inhibitors
29.	Antibiotics
Contact to (health-impaired) individuals/ hospitalization	
30.	Children younger than 16 years live in the same household
31.	Contact to a human or animal with the onset of diarrhoea during the last 12 months
32.	Contact to a patient with the onset of diarrhoea (human or animal) during the last 12 months
33.	Hospitalization for at least 1 week during the last 12 months
34.	Contact to a hospitalized human or animal during the last 12 months
35.	Person with chronic disease lives in the household
36.	Person or animal with a previous positive test for *C. difficile* lives in the household

4.3.3.3. Multivariate analysis

For the multivariate analysis variables with $p \leq 0.2$ associated with *C. difficile*-CITP from the univariate analysis were considered as potential risk factors. To select the variables, which were included in the final multivariate logistic model, a stepwise backward removal procedure with a threshold p-value 0.05 was used as implemented in STATA. Thereby, insignificant variables were sequentially removed and missing values in one of the variables were excluded from the model. In order to acquire more accurate estimates for the selected variables regarding odds ratio and 95% confidence intervals, logistic regression with the remaining significant variables was performed for each participant group.

4.4. Microbiological examination

4.4.1. Isolation of *C. difficile*

All faecal samples underwent direct plating and enrichment culture to isolate *C. difficile*. First, 10 ml of *C. difficile* moxalactam/norfloxacin broth (CDMN, Oxoid, SR173) containing 0.1% sodium-taurocholate (SigmaeAldrich, 86339) were inoculated with 1-4 inoculation loops of each sample (approximately 0.5 g). CDMN agar was used for immediate plating with 100 µl of the faecal broth suspension. Incubation at 37° C under anaerobic conditions for inoculated plates lasted 1-3 days and 14-21 days for enrichment cultures, respectively. Additionally, spore selection was performed on enrichment cultures following incubation. Therefore, 900 µl of each enrichment culture were mixed with an equal amount of 99% ethanol (30 min; room temperature), followed by centrifugation (5000 x g, 10 min) The pellet was re-suspended in 200 µl of 0.8% NaCl. 100 µl of this mixture were then inoculated on CDMN agar and incubated under anaerobic conditions (1-3 d, 37° C).

Colonies with typical morphology showing weak-green fluorescence under illumination at 360 nm and L-proline-aminopeptidase test or *C.difficile*-Latex-Test (Oxoid) positive were preliminary identified as *C. difficile*.

4.4.2. Characterization of *C. difficile* isolates

To obtain pure cultures *C. difficile*-suspected colonies had been subcultured on CDMN agar plates for at least three times. Qiagen DNeasy Blood & Tissue Kit™ was used to isolate bacterial DNA. Species identification was confirmed using *cdd3* PCR amplifying parts of a gene encoding for an ABC-transporter, located downstream from the PaLoc.

4.4.2.1. PCR ribotyping

Capillary gel electrophoresis based PCR ribotyping (seq-PCR ribotyping), was performed according to Indra et al. (2008) using an Applied Biosystems 3130 Genetic Analyzer with GeneScan™-600Liz® size marker (Capillary: 36 cm, Gel: POP7). The profiles were analysed using Webribo Database (http://webribo.ages.at) and BioNumerics™ software. Resulting PCR ribotypes were designated according to standard Cardiff nomenclature (e.g. 010, 014/0, 078). If the PCR ribotype profile did not correspond with any reference profile, the suffix "FLI" with a consecutive number was added to the RT with the most similar profile (e.g. 014/0/FLI01).

4.4.2.2. Toxinotyping

According to Rupnik et al. (1998) isolates were toxinotyped using PCR amplifying fragment A3 of the toxin A gene and fragment B1 of the toxin B gene. Moreover, PCR detecting the genes *cdtA* and *cdtB* encoding for the binary toxin was performed, as described in Stubbs et al. (2000).

4.4.2.3. Multi-Locus Variable-number tandem repeat Analysis (MLVA)

MLVA was performed based on the protocol by van den Berg et al. (2007) and modified according to Bakker et al. (2010). Two PCR-products were sequenced for loci $A6_{Cd}$, $B7_{Cd}$, $C6_{Cd}$, $E7_{Cd}$ and $G8_{Cd}$, whereas only one PCR-product was analyzed for loci $F3_{Cd}$ and $H9_{Cd}$ because of its low length variation. Linear regression was applied to correlate the lengths determined by capillary gel electrophoresis and the number of repeats (copy numbers) for each of the seven loci. The analysis of repeat units and clusters was performed using BioNumerics™ software applying the categorical coefficient and unweighted pair group method using arithmetic averages (UPGMA).

4.4.2.4. Minimum Spanning Tree

A minimum spanning tree (MST) was created to visualize und determine the genetic relationship among isolates of animal and human origin based on the data obtained through MLVA and grouped into RTs. MST was constructed according to the protocol by Marsh et al. (2011) and Schneeberg et al. (2013a) on the basis of the summed tandem-repeat differences (STRD) for all seven MLVA loci. The coefficients were calculated using BioNumerics™ software applying an activated legacy mode, the Manhattan coefficient with an offset "0" and saturation "1,000", and no cross-link distance. For the creation of complexes a maximum neighbour distance with 10 changes and a minimum size of two types was defined. Additionally, the priority rules were set with default settings with first link types that have: (1) maximum number of SLV's, (2) a maximum number of SLV's and DLV's, and (3) a maximum number of entries. Clonal clusters were created if the STRD was ≤ 2; isolates with a STRD of > 2 and ≤ 10 were defined as genetically related clusters (Schneeberg et al. 2013a; Bakker et al. 2010; Marsh et al. 2010; Koene et al. 2012).

5. Results

5.1. Study participant recruitment

During the 14-months-period of participant recruitment from July, 4th 2012 until August, 19th 2013, a total of 851 sampling packages were handed out. Those packages were individually prepared for a total of 2,767 participants (1,160 humans, 1,607 animals) living in 851 different households. 427 of the 851 households, which received sampling packages, participated in the study, resulting in an overall return rate of 50.2%.

The return rate differed considerably among the various acquisition routes (Table 3). Particularly successful was contacting via mailing lists, telephone and social media as well as contacting via cat breeders and kennel clubs with return rates of 76.3% (71/93), 64.4% (38/59), and 62.4% (53/85), respectively. Comparably low return rates were achieved when potential participants had been contacted through general practitioners and veterinarians or in veterinary clinics with 33.6% (51/152) or when sampling packages were handed out via contacts not applicable for any of the listed categories with 12.1% (13/107).

Table 3: Return rates on various participant recruitment routes

Contacts	No. of outgoing sampling packages	No. of participating households	Return rate (in %)
cat breeders	59	38	64.4
dog kennel clubs	85	53	62.4
E-Mail, telephone, social media	93	71	76.3
private	101	55	54.5
colleagues and research institutes (e.g. GNC)	193	111	57.5
symposia, congresses etc.	61	35	57.4
veterinary clinics, veterinary and general practitioners	152	51	33.6
others	107	13	12.1
in total	**851**	**427**	**50.2**

In total, 415 of the 427 participating households (97.2%) could be included in the study. Twelve of the 427 households (2.8%) did not meet the inclusion criteria for the following reasons: missing questionnaires and/or faecal sample(s) of participating household members, consent forms were not completed, or ID numbers had been obviously mixed up by different household members.

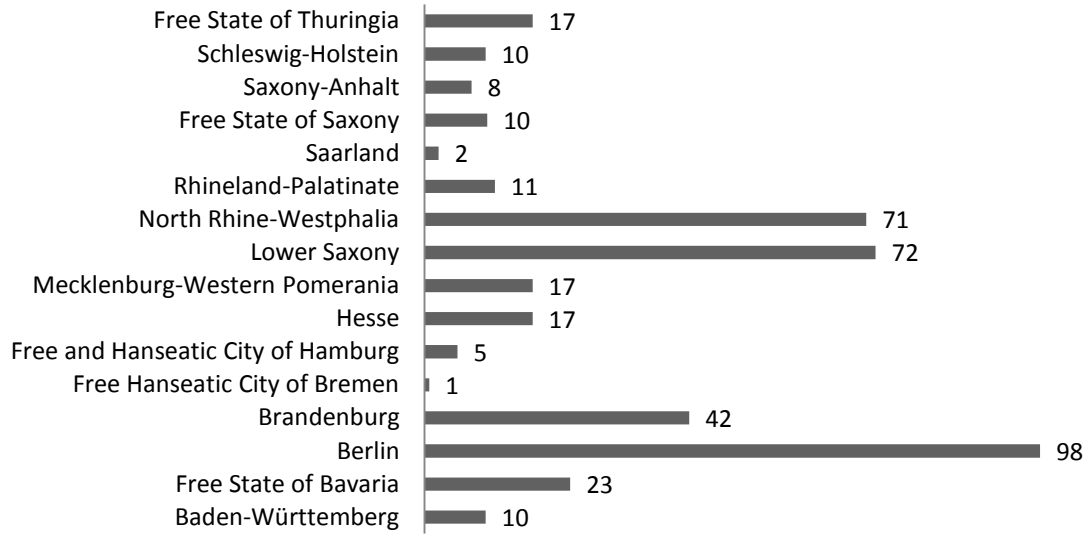
The structure of the 415 households, regarding the number of participating animal owners and their dogs and cats, varied significantly. In total, 35 different combinations of animal-owner-ratios participated. The most frequent composition with one participating owner and one animal occurred in 45.8% (190/415) of the households. Other participant compositions were manifold, with one owner and two animal participants (10.8%; 45), two owners and one animal (9.6%; 40), and two owners and two animals (8.7%; 36) being the most common. The household with the highest number of participants was composed of three owners and ten animals (0.2%;1).

In total, 1,447 faecal samples were collected; 862 (59.6%) were of animal and 585 (40.4%) of

human origin. 29 of 1,447 (2.0%) samples did not meet the inclusion criteria for the following reasons: more than one sample from the same participant or the household had to be excluded due to the reasons described above. A total of 1,418 faecal samples were included in the study with 59.2% (840) being of animal and 40.8% (578) of human origin.

The 1,418 participants belonging to the 415 different households included in the study were distributed throughout all 16 federal states of Germany (Figure 9). The majority of participants could be recruited in Berlin with 23.6% (98/415), followed by Lower Saxony (17.3%; 72/415), North Rhine-Westphalia (17.1%; 71/415), and Brandenburg (10.1%; 42/415).

Figure 9: Distribution of participating households in the federal states of Germany

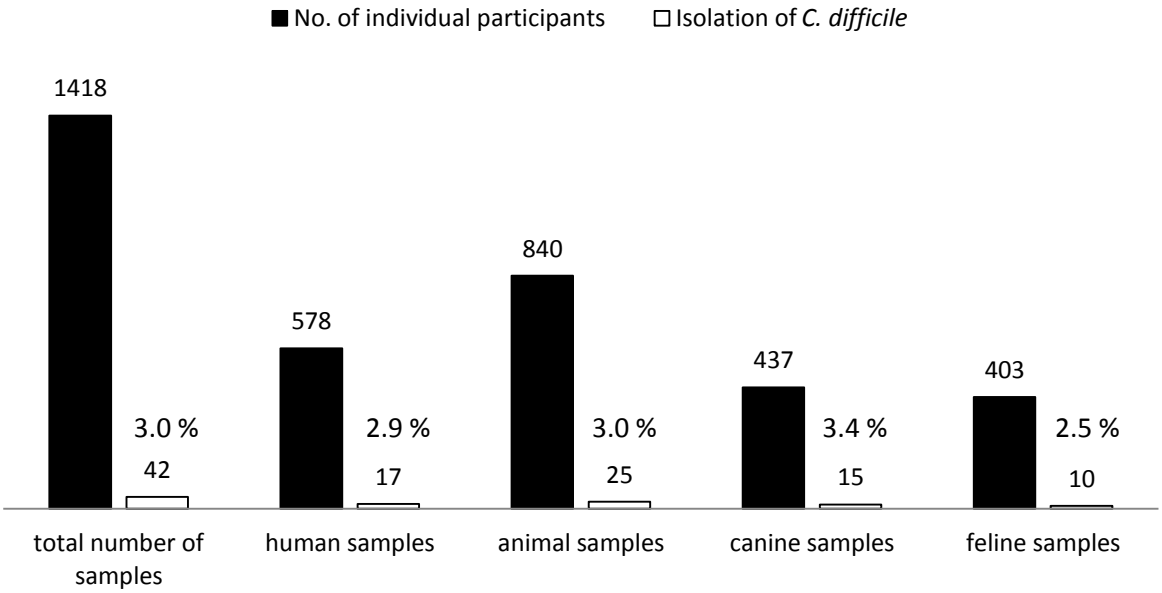


5.2. Microbiological examination

5.2.1. *C. difficile* isolation rates

In total, 1,418 faecal samples with 59.2% (840) of animal and 40.8% (578) of human origin were included for further assessment. The 840 animal samples originated from 437 (52.0%) dogs and 403 (48.0%) cats. *C. difficile* was isolated from 42/1,418 (3.0%) faecal samples with isolation rates of 3.0% (25/840) for animal and 2.9% (17/578) for human samples (Figure 10). All in all, 15 of the 437 (3.4%) canine samples and 10 of the 403 (2.5%) feline samples were positive for *C. difficile*.

Figure 10: Isolation rates of *C. difficile* in different participant groups



C. difficile was detected in 9 out of 25 (36.0%) animal samples by direct plating as well as after enrichment culture. 13 out of 25 (52.0%) samples were positive for *C. difficile* only after enrichment culture, whereas in 3 (12.0%) samples *C. difficile* could only be detected by direct plating. In the faecal samples originating from humans 8 out of 17 (47.1%) samples were positive for *C. difficile* after enrichment culture and direct plating. 7 (41.2%) samples were only after enrichment culture and 2 (11.8%) samples only after direct plating *C. difficile* positive.

5.2.2. Characterization of *C. difficile* isolates

5.2.2.1. PCR-ribotyping

The 43 *C. difficile* isolates of animal origin were assigned to eight different PCR ribotypes (RTs) (001/5/FLI01, 009, 010, 014/0, 014/0/FLI01, 027, 039, 078). Canine samples harboured six different RTs (009, 010, 014/0, 027, 039, 078) and feline samples five different RTs (001/5/FLI01, 010, 014/0, 014/0/FLI01, 039). From one canine sample (participant ID "0934T1") two *C. difficile* strains corresponding with different RTs (RTs 010 and 039) were isolated. However, if more than one isolate was detected in the same faecal sample but was assigned to an identical RT (and MLVA proved no significant differences), they were evaluated as one. Consequently, in animal faecal samples 26 different isolates of *C. difficile* were verified (Table 4). The predominant RT was RT 014/0, which was determined for 10 out of 26 (38.5%) isolates. RT 010 was found in 5 out of 26 (19.2%) isolates, whereas RTs 001/5/FLI01 and 039 were detected in 3 (11.5%) samples each. RTs 014/0/FLI01, 027 and 078 were detected in one (3.8%) isolate each. The RTs 027 and 078 isolates, which are often described as highly pathogenic for humans, originated from canine participants. Interestingly, within two households identical RTs were isolated from two animals (in both cases cats harbouring RT 014/0); further characterization was achieved applying MLVA.

Table 4: *C. difficile* isolation and characterization results

	in total	human	animal	dogs	cats
No. of considered samples	1,418	578	840	437	403
No. of positive samples (%)	42 (3.0)	17 (2.9)	25 (3.0)	15 (3.4)	10 (2.5)
No. of *C. difficile* isolates*[i]	44	18*[h]	26*[a]	16*[a]	10
No. of different RTs	17	12	8	6	5

*[i]: the same RT in one faecal sample was evaluated as one isolate
*[h]: two different RTs were isolated in sample 0748M2
*[a]: two different RTs were isolated in sample 0934T1

All 33 human *C. difficile* isolates belonged to a total of 12 different RTs (003, 003/FLI02, 009/FLI01, 010, 010/FLI01, 014/0, 014/5, 020, 070, 078, 087, 441/FLI01). In one human sample two different RTs (RTs 003 and 078) were detected. As in animal samples, more than one isolate assigned to the same RT in a faecal sample from the same participant was evaluated as only one isolate. Thus, 18 isolates were verified in human faecal samples. The most prevalent human RT was RT 014/0, which was ascertained in 22.2% (4/18) of the human isolates. RT 078 was identified for 3 out of 18 (16.7%) and RT 003 for 2 out of 18 (11.1%) isolates. RTs 003/FLI02, 009/FLI01, 010, 010/FLI01, 014/5, 020, 070, 087 and 441/FLI01 were determined for one (5.6%) isolate each.

No parallel occurrence of *C. difficile* in dogs or cats and their owners living in the same household was detected. However, three RTs were found in human and animal participants as well (RTs 010, 014/0, and RT 078). Besides, RT 078 was only isolated from animal owners and dogs but not from cats.

5.2.2.2. Toxinotyping

Five of the eight (62.5%) RTs, which had been detected in animal samples, were toxigenic; they belonged to RTs 001/5/FLI01, 014/0, 014/0/FLI01, 027, and 078. Regarding the number of isolates originating from animal participants, toxin genes had been detected in 61.5% (16/26). Within toxin-positive isolates, 87.5% (14/16) harboured *tcdA* and *tcdB* genes (encoding for toxins A and B) with additional 12.5% (2/16) also yielding positive PCR results for genes *cdtA* and *cdtB* (encoding for the binary toxin) (Table 5). Interestingly, only 50% (8/16) of the canine *C. difficile* isolates harboured toxin genes, while 80% (8/10) of the strains isolated from cats were toxigenic. In comparison, eight out of twelve (66.7%) RTs isolated from human participants were toxigenic; those were RTs 003, 003/FLI02, 014/0, 014/5, 020, 070, 078, and 087. However, regarding the number of isolates originating from human participants 14 out of 18 (77.8%) harboured toxin genes with 78.6% (11/14) positive for *tcdA* and *tcdB* genes and additional 21.4% (3/14) also being positive for *cdtA* and *cdtB.*

Table 5: Ribotypes and detection of toxin genes in *C. difficile* isolates

PCR-Ribotype	toxin genes				sample origin			in total
	tcdA	*tcdB*	*cdtA*	*cdtB*	human	dog	cat	
001/5/FLI01	+	+	-	-	0	0	3	**3**
003	+	+	-	-	2	0	0	**2**
003/FLI02	+	+	-	-	1	0	0	**1**
009	-	-	-	-	0	2	0	**2**
009/FLI01	-	-	-	-	1	0	0	**1**
010	-	-	-	-	1	4	1	**6**
010/FLI01	-	-	-	-	1	0	0	**1**
014/0	+	+	-	-	4	6	4	**14**
014/0/FLI01	+	+	-	-	0	0	1	**1**
014/5	+	+	-	-	1	0	0	**1**
020	+	+	-	-	1	0	0	**1**
027	+	+	+	+	0	1	0	**1**
039	-	-	-	-	0	2	1	**3**
070	+	+	-	-	1	0	0	**1**
078	+	+	+	+	3	1	0	**4**
087	+	+	-	-	1	0	0	**1**
441/FLI01	-	-	-	-	1	0	0	**1**
total					**18**	**16**	**10**	**44**

5.2.2.3. MLVA

MLVA was applied to further characterize all 44 *C. difficile* isolates as this highly discriminatory typing method enables clonal analysis of strains belonging to the same RT. (Table 6 and Table 7).

Table 6: MLVA results for animal *C. difficile* isolates

Participant IDs		Iso-lation	PCR-RT	MLVA results						
ID	FLI - ID			$A6_{Cd}$	$B7_{Cd}$	$F3_{Cd}$	$H9_{Cd}$	$G8_{Cd}$	$E7_{Cd}$	$C6_{Cd}$
0231T1	12S0599	EC	014/0	23	22	4	2	7	6	32
0248T1	12S0490	DP	014/0	26	19	4	2	6	6	30
0269T1	12S0600	EC	010	14	18	4	2	11	8	43
0382T1	12S0735 w.v. 21.2.	EC	027	27	8	4	2	16	10	30
0570T2	13S0376	DP	001/5/FLI01	15	1	5	2	7	6	31
0652T1	13S0239	EC	014/0	23	15	4	2	7	6	21
0672T1	13S0272 v. 8.5.	EC	078	37	18	4	2	9	8	36
0673T1	13S0240	DP	010	43	20	5	1	11	2	31
0730T1	13S0326	DP	009	27	16	4	2	12	5	40
0762T1	13S0491	DP	014/0	15	1	5	2	7	6	31
0765T2	13S0596	EC	001/5/FLI01	28	14	4	2	7	6	9
0770T2	13S0613	EC	014/0	28	14	4	2	7	6	9
0770T4	13S0614	EC	014/0	14	19	5	1	10	2	17
0773T2	13S0378	DP	009	34	14	4	2	7	6	29
0783T4	13S0490	EC	014/0/FLI01	0	19	4	2	9	8	31
0824T1	13S0608	DP	039	46	21	7	1	1	2	30
0829T5	13S0594	EC	001/5/FLI01	15	1	5	2	7	6	31
0831T1	13S0566	EC	014/0	32	21	4	2	9	6	24
0837T3	13S0592	EC	014/0	33	20	4	2	7	6	22
0838T1E	13S0569	EC	010	22	16	4	2	12	9	26
0895T1	13S0675	EC	010	33	18	4	2	9	8	43
0919T2	13S0782	EC	014/0	32	20	4	2	8	6	31
0919T3	13S0783	EC	014/0	32	19	4	2	8	6	30
0920T1	13S0779	EC	039	34	21	7	1	1	2	31
0934T1	13S0747	DP	039	36	19	7	1	1	2	35
	13S0747	EC	010	39	19	4	2	10	7	40

DP: direct plating; EC: enrichment culture

Table 7: MLVA results for human *C. difficile* isolates

Participant IDs		Iso-lation	PCR-RT	MLVA results						
ID	**FLI - ID**			**A6$_{Cd}$**	**B7$_{Cd}$**	**F3$_{Cd}$**	**H9$_{Cd}$**	**G8$_{Cd}$**	**E7$_{Cd}$**	**C6$_{Cd}$**
0199M1	13S0046	DP	009/FLI01	17	16	4	2	0	8	16
0203M1	13S0330	EC	078	9	20	4	2	9	8	33
0212M1	13S0045	DP	010	35	16	4	2	9	8	40
0416M1	13S0375	DP	070	36	10	5	1	6	6	44
0435M1	13S0131w.v. 25.4.	EC	078	0	19	4	2	9	8	31
0674M1	13S0494	EC	010/FLI01	34	16	4	2	9	6	39
0715M1	13S0570	DP	014/0	27	13	4	2	7	6	26
0718M1	13S0593	DP	014/0	26	18	4	2	7	7	33
0721M2	13S0493	DP	020	23	12	4	2	12	5	22
0748M2	13S0325	DP	003	17	20	4	1	2	9	26
	13S0325a	EC	078	0	19	4	2	9	8	31
0798M1	13S0492	DP	003/FLI02	44	19	5	1	4	13	33
0810M1	13S0525	EC	087	28	10	5	1	10	6	48
0858M1	13S0612	EC	441/FLI01	22	9	4	2	0	8	11
0926M3	13S0748	DP	003	16	20	4	1	2	10	47
0947M2	13S0784	DP	014/5	33	24	4	2	9	4	42
0818M2	13S0639	EC	014/0	19	18	4	2	7	6	27
0456M1	13S0127	EC	014/0	24	20	4	2	7	6	45

DP: direct plating; EC: enrichment culture

Although no parallel occurrence of *C. difficile* in animal owners and their dogs or cats was detected in the study presented here, animals sharing the same household were found to be *C. difficile*-positive at the same time. In two independent households (corresponding with the household ID 0770 and 0919) identical RTs were isolated from two animals (in both cases cats which harboured RT 014/0). Further characterization applying MLVA proved that the strains isolated from the feline participants with the IDs "0770T2" and "0770T4" had no STDR (STRD relating to the previous isolate; Table 8). The partner cats with the IDs "0919T2" and "0919T3" had a STRD of 2. Hence, in both cases the strains isolated within one household can be considered as being clonally related, due to the fact that a STDR of ≤ 2 defines a clonal cluster resp. complex (Bakker et al. 2010; Koene et al. 2012).

Table 8: Characteristics of two *C. difficile* pairs isolated from four cats from two independent households

Participant ID	**RT**	**MLVA**							**STRD**	**Toxin genes**				
		A6*Cd*	**B7*Cd***	**F3*Cd***	**H9*Cd***	**G8*Cd***	**E7*Cd***	**C6*Cd***		*tcdA*	*tcdB*	*cdtA*	*cdtB*	*cdd3*
0770T2	014/0	28	14	4	2	7	6	9		1	1	0	0	1
0770T4	014/0	28	14	4	2	7	6	9	0	1	1	0	0	1
0919T2	014/0	32	20	4	2	8	6	31		1	1	0	0	1
0919T3	014/0	32	19	4	2	8	6	30	2	1	1	0	0	1

5.2.2.4. Minimum spanning tree

Minimum-spanning-tree analysis of the MLVA data of 44 *C. difficile* strains isolated from small companion animals and their owners revealed 7 complexes of related types (STRD ≤ 10) (Figure 11). The largest complex consisted of 8 MLVA types, which corresponded to RTs 014/0 and 014/0/FLI01 with four types originating from canine hosts, three from feline hosts and one from a human host. Another complex, which consisted of two identical MLVA types, which corresponded to RTs 014/0, represented the two strains isolated from partner cats within one household. The second largest complex comprised of 5 MLVA types, which corresponded to RTs 010 and 010/FLI01 with two types originating from canine participants, one from a feline and another two from human participants. Another clonal complex consisted of three identical types belonging to RT 078, whereas two isolates were detected from humans and one isolate originated from a canine study participant. Additionally, one clonal complex comprised three feline isolates belonging to a newly discovered RT 001/5/FLI01 originating from independent households. Another genetically related cluster was rather heterogeneous and comprised a canine RT 014/0 strain and a human isolate belonging to RT 020. Two canine isolates belonging to RT 039 represented the remaining cluster.

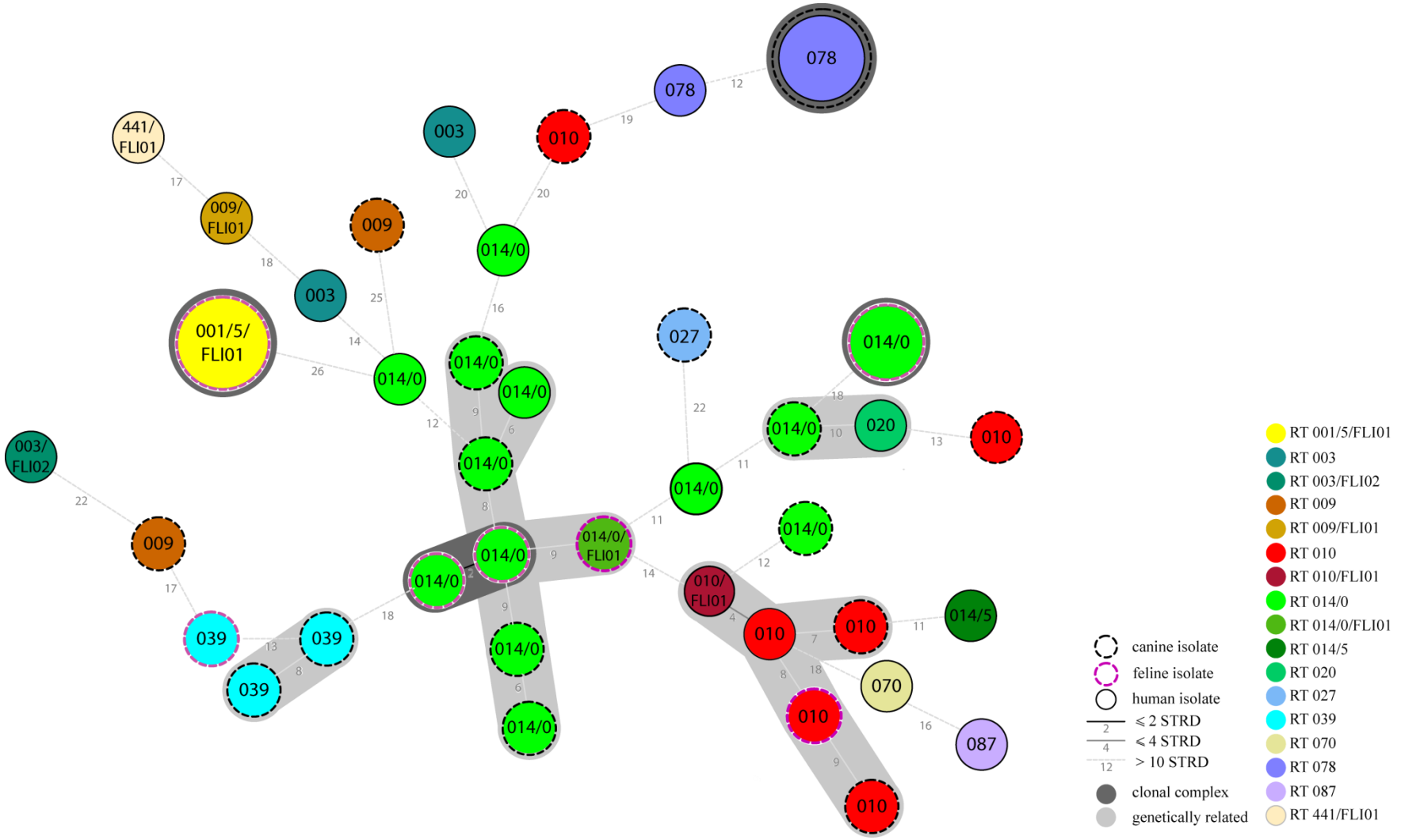


Figure 11: Minimum spanning tree of 44 *C. difficile* isolates originating from animal and human participants typed by multiple-locus variable number tandem-repeat analysis (MLVA).
A total of seven loci have been determined with each circle representing either a unique isolate or isolates that are 100% homologous and belong to a clonal complex. Clonal complexes are defined as isolates that differ from each other with ≤ 2 STRD and are enveloped in a dark grey shade. Isolates that are genetically related are defined with a STRD of ≤ 10 (enveloped in a light grey shade). The STRD between distinct isolates is displayed underneath the lines connecting the circles (fat black line = ≤ 2 STRD, grey line = ≤ 10 STRD, and dashed grey line = > 10 STRD). The origin of the isolate is reflected in the outline of the circle (human isolate = black circle, canine isolate = dashed black circle, feline isolate = dashed pink circle). The PCR ribotype of each isolate is depicted in each circle with a colour coding according to the legend.

5.3. Risk assessment

Sensitivity analyses of four different models included: (1) 190 animals and 190 animal owners; (2) 407 animals and 407 owners; (3) 507 animals and 507 owners; and (4) 840 animals and 578 owners. However, no clustering or significant increase in performance of model (1) to (3) in comparison to model (4) could be detected. Hence, all participants who met the inclusion criteria were included in the statistical analysis of potential risk factors associated with *C. difficile*-CITP.

5.3.1. Univariate analysis

5.3.1.1. Faecal shedding of *C. difficile* in dogs and cats

The univariate analysis for animal participants was performed for 47 variables (Table 1, chapter 4.3.3.1) including six demographic/animal housing parameters, two factors related to "contact to other animals", seven "contact between animal and owner "-related variables, seven factors related to "stay in different sites", eight feed consumption parameters, six health status factors, three medication-related variables and eight parameters related to "contact to health-impaired individuals/ hospitalization".

In total, ten variables belonging to five different categories proved to be significantly associated with *C. difficile*-CITP in animals (Table 10): (1) one demographic parameter (being five to nine years old (OR 3.53, 95% CI 1.25-10.04, $p = 0.018$)); (2) three health status factors (inappetence (OR 6.70, 95% CI 2.34-19.14, $p < 0.001$), acute disease (OR 6.16, 95% CI 2.43-15.62, $p < 0.001$), and diarrhoea during the last four weeks prior faecal sampling (OR 2.73, 95% CI 1.13-6.57, $p = 0.025$)); (3) two medication related variables (regular intake of proton pump inhibitors (OR 25.43, 95% CI 6.68-96.85, $p < 0.001$) and antibiotics, at least once during the last 3 months prior sampling (OR 4.37, 95% CI 1.95-9.77, $p < 0.001$)); (4) two parameters related to "contact to health-impaired individuals/ hospitalization" (contact to a human with symptoms of diarrhoea (OR 3.25, 95% CI 1.18-8.90, $p = 0.022$), owner suffers from a chronic disease (OR 3.64, 95% CI 1.44-9.17, $p = 0.006$)); and (5) one feed consumption parameter (dry feed (OR 0.13, 95% CI 0.06-0.29, $p < 0.001$)). In contrast to the other variables, the latter parameter was negatively associated with *C. difficile*-CITP.

Table 9: Univariate analysis for faecal shedding of *C. difficile* in dogs and cats

	CD positive	CD negative	n	*p*-Value	OR	95% CI
Demographic factors and animal housing						
Cat (dog)	10 (15)	393 (422)	840	0.420	0.72	0.32-1.61
Female (male)	12 (13)	460 (353)	838	0.396	0.71	0.32-1.57
Neutered (not neutered)	13 (12)	398 (406)	829	0.806	1.11	0.50-2.45
Age in years			840			
< 1	1	44	45	0.651	1.65	0.19-14.45
1-4	5	363	368	Ref.	.	.
5-9	13	267	280	0.018	3.53	1.25-10.04
10-22	6	141	147	0.066	3.09	0.93-10.28
Animal housing Dog: house/flat (kennel/garden/ stable)	14 (1)	361 (50)	426	0.527	1.94	0.25-15.06
Cat: house/flat (outdoor)	6 (4)	290 (85)	385	0.211	0.44	0.12-1.59
Contact between animal and owner						
The animal is allowed to…						
… lie on the couch	22 (3)	671 (139)	835	0.502	1.52	0.45-5.15
… sleep in bed	18 (7)	521 (285)	831	0.450	1.41	0.58-3.41
… be washed in the tub/shower	16 (9)	352 (443)	820	0.057	2.24	0.98-5.12
… be petted	25 (0)	808 (1)	834	.	.	.
… feed out of the hand	24 (1)	749 (43)	817	0.756	1.38	0.18-10.43
… lick the owner's face	18 (7)	471 (319)	815	0.219	1.74	0.72-4.22
other contacts	9 (3)	148 (165)	325	0.074	3.34	0.89-12.59
Contact to other animals						
Regular contact to other farm or companion animals						
Contact	20 (4)	720 (88)	832	0.379	0.61	0.20-1.83
Dogs	13 (11)	505 (302)	831	0.404	0.71	0.31-1.60
Cats	13 (11)	458 (349)	831	0.801	0.90	0.40-2.03
Sheep	0 (24)	17 (790)	831	.	.	.
Poultry	1 (23)	74 (733)	831	0.413	0.43	0.06-3.23
Wild animals	0 (24)	33 (774)	831	.	.	.
Small mammals	2 (22)	52 (755)	831	0.712	1.32	0.30-5.77
Horses	1 (23)	73 (734)	831	0.421	0.44	0.06-3.28
Cattle	0 (24)	14 (793)	831	.	.	.
Pigs	0 (24)	4 (803)	831	.	.	.
Others	1 (23)	47 (760)	831	0.733	0.70	0.09-5.32

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	CD positive	CD negative	n	*p*-Value	OR	95% CI
Contact to other animals *(continued)*						
Regular contact to infant animals during the last 12 months						
Contact	8 (17)	349 (449)	823	0.248	0.61	0.26-1.42
Dogs	4 (21)	192 (606)	823	0.356	0.60	0.20- 1.77
Cats	4 (21)	172 (626)	823	0.507	0.69	0.23- 2.05
Sheep	0 (25)	8 (790)	823	.	.	.
Poultry	1 (24)	8 (790)	823	0.191	4.11	0.49- 34.21
Wild animals	0 (25)	2 (796)	823	.	.	.
Small mammals	1 (24)	9 (789)	823	0.228	3.65	0.44- 29.99
Horses	0 (25)	4 (794)	823	.	.	.
Cattle	0 (25)	4 (794)	823	.	.	.
Pigs	0 (25)	1 (797)	823	.	.	.
Others	0 (25)	3 (795)	823	.	.	.
Stay in different sites during the last 12 months						
Boarding kennel	2 (23)	42 (773)	840	0.533	1.60	0.37-7.02
Animal shelter	0 (25)	12 (803)	840	.	.	.
Kindergarten/school	0 (25)	10 (805)	840	.	.	.
Dog-/cat-show	6 (19)	154 (661)	840	0.524	1.36	0.53-3.45
Health care/ rehabilitation facility	0 (25)	19 (796)	840	.	.	.
In use as therapy dog	0 (25)	15 (800)	840	.	.	.
Pet obedience school	1 (24)	134 (681)	840	0.130	0.21	0.03-1.58
Feed consumption						
Canned feed	15 (10)	573 (242)	840	0.272	0.63	0.28-1.43
Dry feed	14 (11)	741 (74)	840	<0.001	0.13	0.06-0.29
Jerky	7 (18)	263 (552)	840	0.653	0.82	0.34-1.98
Raw meat (products)	9 (16)	303 (512)	840	0.904	0.95	0.41-2.18
Leftovers	5 (20)	183 (632)	840	0.772	0.86	0.32-2.33
Dog/cat treats	15 (10)	592 (223)	840	0.170	0.57	0.25-1.28
Animal feed additives	6 (19)	250 (565)	840	0.477	0.71	0.28-1.81
Others	6 (19)	202 (613)	840	0.929	0.96	0.38-2.43

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	CD positive	CD negative	n	*p*-Value	OR	95% CI
Health status/ behaviour						
Coprophagy	1 (24)	118 (675)	818	0.162	0.24	0.03-1.78
Polyphagia	2 (23)	102 (683)	810	0.468	0.58	0.14-2.51
Inappetence	5 (20)	28 (750)	803	<0.001	6.70	2.34-19.14
Acute disease	7 (16)	53 (746)	822	<0.001	6.16	2.43-15.62
Chronic disease	6 (19)	137 (651)	813	0.395	1.50	0.59-3.83
Diarrhoea during the last 4 weeks	8 (15)	127 (650)	800	0.025	2.73	1.13-6.57
Medication						
Anti-inflammatory drugs (regular intake)	3 (21)	34 (772)	830	0.067	3.24	0.92-11.41
Proton pump inhibitors (regular intake)	4 (21)	6 (801)	832	<0.001	25.43	6.68-96.85
Antibiotics (at least once during the last 3 months prior sampling)	12 (13)	141 (667)	833	<0.001	4.37	1.95-9.77
Contact to health-impaired individuals/ hospitalization						
Contact to a human or animal with the onset of diarrhoea during the last 12 months						
Human or animal	13 (4)	358 (251)	626	0.154	2.28	0.73-7.07
Human	11 (6)	214 (379)	610	0.022	3.25	1.18-8.90
Animal	6 (11)	213 (379)	609	0.954	0.97	0.35-2.66
Owner of the tested pet has suffered from diarrhoea during the last 4 weeks	6 (14)	86 (399)	505	0.171	1.99	0.74-5.32
Hospitalization during the last 12 months in a veterinary clinic	3 (22)	42 (759)	826	0.156	2.46	0.71-8.56
Contact to a hospitalized human or animal during the last 12 months						
Human or animal	10 (13)	257 (457)	737	0.464	1.37	0.59-3.16
Human	6 (17)	187 (527)	737	0.991	0.99	0.39-2.56
Animal	4 (19)	98 (616)	737	0.617	1.32	0.44-3.97
Person with a chronic disease lives in the household	9 (9)	135 (332)	485	0.062	2.46	0.96-6.33
Owner of the tested pet suffers from a chronic disease	10 (9)	113 (370)	502	0.006	3.64	1.44-9.17
Person or animal with a previous positive test for *C. difficile* lives in the household	1 (8)	12 (380)	401	0.211	3.96	0.46-34.21
Owner of the participating pet was previously tested positive for *C. difficile*	0 (16)	1 (437)	454	.	.	.

CD: *C. difficile* isolation; Ref.: reference category; OR: odds ratio; CI: confidence interval.
C*omment*: bracketed data indicate the number of participants not applying to the variable in row.

5.3.1.2. Distinctive characteristics for dogs

The univariate analysis for factors associated with *C. difficile*-CITP in dogs showed certain distinctive characteristics compared to the univariate analysis for all animal participants (Table 10. Generally, 437 dogs were included in the statistical analysis. In total, seven variables belonging to four different categories proved to be significantly associated with *C. difficile*-CITP in dogs: (1) contact to other animals (regular contact to other farm or companion animals (OR 0.12, 95% CI 0.04-0.42, $p < 0.001$)); (2) feed consumption (dry feed (OR 0.10, 95% CI 0.03-0.30, $p < 0.001$)); (3) health status/behaviour (inappetence and acute disease (OR 7.23, 95% CI 2.11-24.84, $p = 0.002$ resp. OR 5.69, 95% CI 1.78-18.19, $p = 0.003$)); and (4) medication (regular intake of anti-inflammatory drugs and proton pump inhibitors (OR 4.64, 95% CI 1.21-17.78, $p = 0.025$ resp. OR 29.89, 95% CI 7.05-126.75, $p < 0.001$), and treatment with antibiotics at least once during the last three months prior sampling (OR 4.15, 95% CI 1.46-11.80, $p = 0.008$)). In contrast to the other variables, the regular contact to other farm or companion animals and consumption of dry feed were negatively associated with *C. difficile*-CITP.

Table 10: Distinctive characteristics from the univariate analysis for faecal shedding of *C. difficile* in dogs

	CD positive	CD negative	n	*p*-Value	OR	95% CI
Contact to other animals						
Regular contact to other farm or companion animals						
Contact	11 (4)	404 (18)	437	< 0.001	0.12	0.04-0.42
Feed consumption						
Dry feed	6 (9)	366 (56)	437	<0.001	0.10	0.03-0.3
Health status/ behaviour						
Inappetence	4 (11)	19 (378)	415	0.002	7.23	2.11-24.84
Acute disease	5 (8)	41 (373)	427	0.003	5.69	1.78-18.19
Medication						
Anti-inflammatory drugs (regular intake)	3 (11)	23 (391)	428	0.025	4.64	1.21-17.78
Proton pump inhibitors (regular intake)	4 (11)	5 (411)	431	<0.001	29.89	7.05-126.75
Antibiotics (at least once during the last 3 months prior sampling)	7 (8)	73 (346)	434	0.008	4.15	1.46-11.80

CD: *C. difficile* isolation; Ref.: reference category; OR: odds ratio; CI: confidence interval.
C*omment*: bracketed data indicate the number of participants not applying to the variable in row.

5.3.1.3. Distinctive characteristics for cats

Similar to the distinctive characteristics for factors associated with CITP of *C. difficile* in canine participants, the univariate analysis showed certain differences for feline participants compared to the univariate analysis for all animal participants (Table 11). In total, 403 cats were included in the statistical analysis.

Five variables from four different categories remained in the univariate model for significant *C. difficile* risk factors in cats: (1) feed consumption (dry feed (OR 0.19, 95% CI 0.04-0.97, $p < 0.046$)); (2) health status/behaviour (acute disease (OR 7.77, 95% CI 1.49-40.57, $p = 0.015$)); (3) medication (antibiotic treatment at least once during the last three months prior sampling (OR 4.72, 95% CI 1.33-16.76, $p = 0.016$)); (4) contact to health-impaired

individuals/ hospitalization (contact to a human with the onset of diarrhoea during the last twelve months (OR 5.31, 95% CI 1.08-25.95, $p = 0.039$) and contact to a hospitalized animal during the last twelve months (OR 4.05, 95% CI 1.10-14.87, $p = 0.035$)). In contrast to canine participants, no feline participant with a positive microbiological examination for the presence of *C. difficile* had been previously treated with anti-inflammatory drugs or proton pump inhibitors.

Table 11: Distinctive characteristics from the univariate analysis for faecal shedding of *C. difficile* in cats

	CD positive	CD negative	n	*p*-Value	OR	95% CI
Feed consumption						
Dry feed	8 (2)	375 (18)	403	0.046	0.19	0.04-0.97
Health status/ behaviour						
Acute disease	2 (8)	12 (373)	395	0.015	7.77	1.49-40.57
Medication						
Antibiotics (at least once during the last 3 months prior sampling)	5 (5)	57 (312)	399	0.016	4.72	1.33-16.76
Contact to health-impaired individuals/ hospitalization						
Contact to a human or animal with the onset of diarrhoea during the last 12 months						
Human	7 (2)	126 (191)	326	0.039	5.31	1.08-25.95
Contact to a hospitalized human or animal during the last 12 months						
Animal	4 (6)	50 (304)	364	0.035	4.05	1.10-14.87

CD: *C. difficile* isolation; Ref.: reference category; OR: odds ratio; CI: confidence interval.
C*omment*: bracketed data indicate the number of participants not applying to the variable in row.

5.3.1.4. Faecal shedding of *C. difficile* in animal owners

The univariate analysis for the animal owners was performed for 36 variables (Table 2, chapter 4.3.3.2) including five demographic parameters, seven factors related to "contact between animal and owner", two "contact to other animals"-related variables, three factors related to "contact between participating dog/cat and others", five food consumption parameters, four factors concerning the health status, three medication-related variables, and seven parameters related to "contact to (health-impaired) individuals/ hospitalization".

In total, five variables belonging to three different categories proved to be significantly associated with *C. difficile*-CITP in animal owners (Table 13): (1) three demographic factors (age of up to twelve months (OR 70.00, 95% CI 5.41-905.30, $p = 0.001$), occupation in other fields than agriculture, food production or health care (OR 0.28, 95% CI 0.09-0.87, $p = 0.028$), and "no current occupation" (OR 3.45, 95% CI 1.31-9.11, $p = 0.013$)); (2) one factor related to contact between participating dog/cat and others (regular contact to poultry (OR 4.52, 95% CI 1.37-14.90, $p = 0.013$)); and (3) one medication-linked parameter (antibiotic treatment at least once during the last two months prior sampling (OR 7.79, 95% CI 2.91-20.87, $p < 0.001$)). A negative correlation was observed for *C. difficile*-positivity and working in other fields than agriculture, food production and health care.

Table 12: Univariate analysis for faecal shedding of *C. difficile* in animal owners

	CD positive	CD negative	n	*p*-Value	OR	95% CI
Demographic factors						
Age in years			566			
<1	2	1	3	0.001	70.00	5.41-905.30
1-4	1	4	5	0.072	8.75	0.82-93.11
5-17	0	26	26	.	.	.
18-44	5	175	180	Ref.		
45-64	7	274	281	0.850	0.89	0.28-2.86
65-87	2	69	71	0.986	1.01	0.19-5.35
Sex						
Female (Male)	10 (7)	384 (171)	572	0.367	0.64	0.24-1.70
Place of residence						
District of Germany	(no significance regarding a certain district of Germany could be distinguished)					
Countryside (large/provincial city)	11 (6)	316 (230)	563	0.575	1.33	0.49- 3.66
Profession/ field of occupation						
Agriculture	1 (16)	21 (540)	578	0.653	1.61	0.20- 12.70
Food production	0 (17)	5 (556)	578	.	.	.
Health care	1 (16)	101 (460)	578	0.225	0.28	0.04-2.17
Other field of action	4 (13)	293 (268)	578	0.028	0.28	0.09-0.87
No current occupation (e.g. retirement, parental leave)	9 (8)	138 (423)	578	0.013	3.45	1.31-9.11
Not specified	15 (2)	519 (42)	578	0.516	0.61	0.13- 2.74
Contact between animal and owner						
The animal is allowed to…						
… lie on the couch	12 (3)	389 (99)	503	0.978	1.02	0.28-3.68
… sleep in bed	9 (6)	298 (186)	499	0.902	0.94	0.33-2.67
… be washed in the tub/shower	8 (7)	209 (269)	493	0.463	1.47	0.52-4.12
… be petted	15 (0)	488 (1)	504	.	.	.
… feed out of the hand	14 (1)	443 (32)	490	0.991	1.01	0.13-7.94
… lick the face	9 (6)	268 (206)	489	0.790	1.15	0.40-3.29
other contacts	5 (4)	84 (133)	226	0.319	1.98	0.52-7.58

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	CD positive	CD negative	n	*p*-Value	OR	95% CI
Contact to other animals						
Animal husbandry (additionally to tested dog/cat)						
Keeping farm or companion animals	9 (8)	342 (219)	578	0.506	0.72	0.27-1.90
Dogs	4 (13)	163 (398)	578	0.622	0.75	0.24-2.34
Cats	6 (11)	199 (362)	578	0.988	0.99	0.36-2.72
Sheep	1 (16)	10 (551)	578	0.252	3.44	0.42-28.54
Poultry	3 (14)	40 (521)	578	0.118	2.79	0.77-10.12
Wild animals	0 (17)	2 (559)	578	.	.	.
Small mammals	1 (16)	38 (523)	578	0.885	0.86	0.11-6.66
Horses	0 (17)	50 (511)	578	.	.	.
Cattle	1 (16)	9 (552)	578	0.215	3.83	0.46-32.09
Pigs	0 (17)	5 (556)	578	.	.	.
Others	1 (16)	56 (505)	578	0.582	0.56	0.07-4.33
Multiple contacts to other animals not in care by participant during the last 12 months						
Contact	12 (5)	417 (144)	578	0.728	0.83	0.29-2.39
Dogs	12 (3)	378 (105)	498	0.872	1.11	0.31-4.01
Cats	8 (7)	270 (213)	498	0.844	0.90	0.32-2.53
Sheep	1 (14)	40 (443)	498	0.823	0.79	0.10-6.17
Poultry	3 (12)	46 (437)	498	0.193	2.38	0.65-8.72
Wild animals	1 (14)	38 (445)	498	0.865	0.84	0.11-6.53
Small mammals	2 (13)	75 (408)	498	0.817	0.84	0.19-3.78
Horses	2 (13)	127 (356)	498	0.273	0.43	0.10-1.94
Cattle	2 (13)	52 (431)	498	0.753	1.28	0.28-5.81
Pigs	1 (14)	40 (443)	498	0.823	0.79	0.10-6.17
Others	1 (14)	38 (445)	498	0.865	0.84	0.11-6.53
Contact between participating dog/cat and others						
Regular contact to other farm or companion animals	14 (1)	420 (64)	499	0.468	2.13	0.28-16.50
Dogs	11 (4)	306 (177)	498	0.433	1.59	0.50-5.07
Cats	10 (5)	239 (244)	498	0.199	2.04	0.69-6.06
Sheep	0 (15)	11 (472)	498	.	.	.
Poultry	4 (11)	36 (447)	498	0.013	4.52	1.37-14.90
Wild animals	0 (15)	25 (458)	498	.	.	.
Small mammals	2 (13)	31 (452)	498	0.302	2.24	0.48-10.39
Horses	0 (15)	56 (427)	498	.	.	.
Cattle	1 (14)	13 (470)	498	0.376	2.58	0.32-21.14

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	CD positive	CD negative	n	*p*-Value	OR	95% CI
Contact between participating dog/cat and others *(continued)*						
Pigs	0 (15)	4 (479)	498	.	.	.
Others	1 (14)	25 (458)	498	0.799	1.31	0.17-10.35
Regular contact to infant animals during the last 12 months						
Contact	7 (8)	156 (322)	493	0.262	1.81	0.64-5.07
Dogs	3 (12)	100 (378)	493	0.931	0.95	0.26- 3.41
Cats	4 (11)	59 (419)	493	0.114	2.58	0.80- 8.37
Sheep	0 (15)	4 (474)	493	.	.	.
Poultry	0 (15)	4 (474)	493	.	.	.
Wild animals	0 (15)	2 (476)	493	.	.	.
Small mammals	0 (15)	5 (473)	493	.	.	.
Horses	0 (15)	4 (474)	493	.	.	.
Cattle	0 (15)	4 (474)	493	.	.	.
Pigs	0 (15)	1 (477)	493	.	.	.
Others	0 (25)	3 (475)	493	.	.	.
Contact to a human or animal with the onset of diarrhoea during the last 12 months						
Human or animal	6 (4)	187 (150)	347	0.778	1.20	0.33-4.34
Human	3 (7)	136 (198)	344	0.500	0.62	0.16-2.46
Animal	5 (5)	93 (240)	343	0.141	2.58	0.73-9.12
Food consumption						
Tap water as cold drink	14 (3)	453 (83)	553	0.809	0.86	0.24-3.04
Raw milk/-products	12 (4)	394 (141)	551	0.903	1.07	0.34-3.38
Raw meat/-products	14 (3)	389 (146)	552	0.384	1.75	0.50-6.18
Ready-to-eat-salads	11 (6)	372 (167)	556	0.706	0.82	0.30-2.26
Probiotics	7 (10)	292 (250)	559	0.306	0.60	0.22-1.60
Health status						
Diarrhoea during the last 4 weeks	3 (14)	106 (451)	574	0.886	0.91	0.26-3.23
Chronic disease	6 (10)	134 (423)	573	0.224	1.89	0.68-5.31
Chemotherapy during the last 12 months	0 (17)	3 (555)	575	.	.	.
Previous positive test for *C. difficile*	0 (14)	2 (500)	516	.	.	.

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	CD positive	CD negative	n	*p*-Value	OR	95% CI
Health status *(continued)*						
Medication						
Anti-inflammatory drugs (regular intake)	3 (14)	81 (475)	573	0.724	1.26	0.35-4.47
Proton pump inhibitors (regular intake)	2 (15)	49 (505)	571	0.679	1.37	0.31-6.18
Antibiotics (at least once during the last 2 months prior sampling)	9 (8)	70 (485)	572	<0.001	7.79	2.91-20.87
Contact to (health-impaired) individuals/ hospitalization						
Children younger than 16 years live in the same household	3 (13)	120 (433)	569	0.778	0.83	0.23- 2.97
Contact to a human or animal with the onset of diarrhoea during the last 12 months						
Human or animal	8 (6)	283 (144)	441	0.480	0.68	0.23-1.99
Human	4 (10)	163 (264)	441	0.469	0.65	0.20-2.10
Animal	7 (7)	177 (250)	441	0.525	1.41	0.49-4.10
Contact to a diarrhoeic patient (human or animal) during the last 12 months						
Human or animal	7 (6)	247 (174)	434	0.728	0.82	0.27-2.49
Human	3 (4)	143 (104)	254	0.434	0.55	0.12-2.49
Animal	7 (0)	152 (95)	254	.	.	.
Hospitalization for at least 1 week during the last 12 months	2 (15)	45 (506)	568	0.598	1.50	0.33-6.76
Contact to a hospitalized human or animal during the last 12 months						
Human or animal	5 (11)	232 (233)	481	0.152	0.46	0.16-1.33
Human	4 (1)	195 (38)	238	0.826	0.78	0.08-7.17
Animal	2 (3)	60 (174)	239	0.476	1.93	0.32-11.85
Person with chronic disease lives in the household	6 (10)	160 (378)	554	0.506	1.42	0.51-3.97
Person or animal with a previous positive test for *C. difficile* lives in the household	0 (13)	15 (426)	454	.	.	.

CD: *C. difficile* isolation; Ref.: reference category; OR: odds ratio; CI: confidence interval.
C*omment*: bracketed data indicate the number of participants not applying to the variable in row.

5.3.2. Multivariate analysis

5.3.2.1. Multivariate analysis for faecal shedding of *C. difficile* in dogs and cats

In the multivariate analysis for independent factors associated with *C. difficile*-CITP in dogs and cats all animal variables with a $p \leq 0.05$ in the univariate analysis were included (Table 13). Thereby, regular medication with PPI (OR 14.82, $p = 0.014$), antibiotic treatment at least once during the last three months prior sampling (OR 4.13; $p = 0.008$), the contact to a human with the onset of diarrhoea during the last twelve months (OR 2.94; $p = 0.048$), and the consumption of dry feed (OR 0.13; $p = 0.001$) remained independently associated with the outcome in our model.

Table 13: Multivariate analysis for faecal shedding of *C. difficile* in dogs and cats

	OR	95% CI	*p*-Value
Consumption of dry feed	0.13	0.04-0.42	0.001
Intake of proton pump inhibitors (regular intake)	14.82	1.73-126.78	0.014
Intake of antibiotics (at least once during the last 3 months prior sampling)	4.13	1.44-11.84	0.008
Contact to a human with diarrhoea during the last 12 months	2.94	1.01-8.60	0.048

5.3.2.1.1. Distinctive characteristics for dogs

Using the selectively derived univariate data for canine participants the regular intake of PPI (OR 46.29, $p < 0.001$) and consumption of dry feed (OR 0.08, $p < 0.001$) remained as independent factors in the final multivariate model (Table 14).

Table 14: Multivariate analysis for faecal shedding of *C. difficile* in dogs

	OR	95% CI	*p*-Value
Intake of proton pump inhibitors (regular intake)	46.29	8.40-255.00	<0.001
Consumption of dry feed	0.08	0.06-0.28	<0.001

5.3.2.1.2. Distinctive characteristics for cats

In contrast to canine participants, independent factors from the multivariate analysis associated with *C. difficile* isolation in cats were the contact to a human with the onset of diarrhoea (OR 7.47, $p = 0.017$) and the contact to a hospitalized animal (OR 6.29, $p = 0.011$) (Table 15); both factors being regarded to a period of the last twelve months prior study participation.

Table 15: Multivariate analysis for faecal shedding of *C. difficile* in cats

	OR	95% CI	*p*-Value
Contact to a human with the onset of diarrhoea during the last 12 months	7.47	1.44-38.76	0.017
Contact to a hospitalized animal during the last 12 months	6.29	1.52-26.09	0.011

5.3.2.2. Multivariate analysis for faecal shedding of *C. difficile* in animal owners

Independent factors for *C. difficile*-CITP in human participants resulted from the multivariate analysis of significant factors ($p \leq 0.05$) derived from the univariate analysis (Table 16).

Significant independent factors associated with *C. difficile* isolation in animal owners regarded having no current occupation (OR 2.83, $p = 0.045$) and the intake of antibiotics (OR 8.62, $p < 0.001$).

Table 16: Multivariate analysis for faecal shedding of *C. difficile* in animal owners

	OR	**95% CI**	***p*-Value**
No current occupation (e.g. retirement, parental leave)	2.83	1.02-7.84	0.045
Intake of antibiotics	8.62	2.93-25.38	<0.001

6. Discussion

C. difficile infections have been on the rise for decades, especially in elderly and immunocompromised patients. However, new ribotypes causing infections in younger persons without obvious risk factors have emerged since 2001. Increasing efforts to find the source of infection enabled to find out that patients are often colonized with the pathogen before developing an infection. Antibiotic therapy has been found to disrupt the intestinal microbiota resulting in the loss of colonization resistance (Kim et al. 2017). Hence, CDI is often a secondary infection induced by antibiotic treatment of an underlying disease. The ability to form spores enables *C. difficile* to survive in different environments and increases the chance for susceptible hosts to acquire *C. difficile*. Orally ingested, viable spores can then germinate and proliferate into vegetative cells within the intestinal environment, consequently, causing colonization or infection. Toxin-producing *C. difficile* are able to cause infections symptoms varying from mild abdominal convulsions and diarrhoea up to life-threatening PMC in humans. Besides human patients, also animals can be affected by *C. difficile*. The isolation of identical RTs from humans and animals led to the assumption of a potential zoonotic transmission of *C. difficile*. Noteworthy, about one quarter of the CDI-cases occurs within the community (Hensgens et al. 2012). Additionally, a high proportion of those CDI-patients has not been exposed to other patients suffering from CDI which strongly suggests a so far undetected source of infection (Eyre et al. 2013a). Those findings have increasingly urged studies on the prevalence of *C. difficile* in various animal species and its zoonotic potential (Keessen et al. 2011b; Arroyo et al. 2005a). To get new insights into the epidemiology of *C. difficile,* this study was conducted with emphasis on the interaction of small companion animals and their owners. For the first time, isolation rates of *C. difficile* had been investigated in a large-scale nation-wide survey. Additionally, *C. difficile* isolates had been molecularly characterized and epidemiological factors for *C. difficile* colonization/infection had been analyzed in dogs, cats and their owners.

6.1. Microbiological examination vs. diagnostical detection of *C. difficile*

In human as well as in veterinary medicine there is an on-going debate on the gold standard for the detection of *C. difficile*. Different detection methods hamper the comparability of analyses and research results (Keessen and Lipman 2012). In 2007, Rodriguez-Palacios et al. detected *C. difficile* in 20% (12/60) of Canadian retail meat samples. Two years later, they described a much lower prevalence of 6.1% (13/214). According to the authors the remarkable difference was caused by limitations such as (1) non-systematic sampling procedures, (2) non-validated culture methods, and (3) a limited study population. In particular reliable culture methods are pivotal prerequisites for epidemiological studies since inadequate detection methods might be responsible for underestimated prevalence rates of *C. difficile* in animals (Blanco et al. 2013). For reliable *C. difficile* detection the authors recommended enrichment in a selective broth prior plating on solid media as previously described by Schneeberg et al. (2013a); Schneeberg et al. (2013b) and as applied in this study as well. Additionally, Weese & Fulford (2011) discussed different approaches of *C. difficile* detection in animal faeces including toxin detection. Nevertheless, a consensus concerning the gold standard is still missing. Since this study aimed in analyzing isolation rates in healthy populations and characterizing *C. difficile* isolates, it was obligatory to apply a culture-dependant detection method.

6.2. Isolation rates and molecular characteristics of *C. difficile* isolates

Comparability of isolation rates in the context of prevalence study designs

Overall isolation rates determined within this thesis were low. However, data comparison is impaired by different study designs in earlier studies. Though, the isolation rates for human

samples were similar to data of Wilcox et al. (2008) who surveyed CA-CDI in England. In a case-control study randomly selected faecal samples were investigated in an urban and a rural area. In both cohorts, 2.1% (42/2000) of the samples were positively tested. Yet, only diarrhoeal faecal samples were examined and subsequently tested for *C. difficile* cytotoxin; thus, impeding the direct comparison of *C. difficile* rates with the results presented here. Nevertheless, the isolation rate of toxigenic strains in the human study population examined here was somewhat comparable with 2.4% (14/584). In a Dutch survey unformed stool samples from 2,423 patients were examined for the presence of toxins A and B and 1.5% (37) of the samples turned out to be positive (Bauer et al. 2009). The majority of the patients who had been tested positive had had no contact to healthcare facilities in the past year. The authors further concluded that CA-CDI is not associated with nosocomial CDI outbreaks as a quarter of the *C. difficile* isolates belonged to new or rare RTs and epidemic RTs such as RT 027 could not be detected. This contradicts the study results presented here since 77.8% (14/18) of the human isolates were toxigenic with 21.4% (3/14) of those belonging to potentially highly virulent RTs additionally producing the binary toxin. Nonetheless, the isolation rate in this study population is in concordance with an estimated asymptomatic carriage rate of 3% in humans within the community (Kern 2010), and higher than the prevalence rate of 1.23% (30/2494) reported in a cross-sectional population-based study conducted in the Netherlands (Zomer et al. 2017).
It is even more challenging to compare the isolation rate for animal participants in this study to similar surveys. Isolation rates in dogs and cats varied between 1% (Weese et al. 2001) and 58% (Lefebvre et al. 2006b) with up to 76% in diarrhoeic dogs (Silva et al. 2013b); yet, study designs and study populations differed significantly mainly focussing on participants attending veterinary health care settings or being kept in animal shelters. Though, in Arizona, USA, 197 canine faecal samples were collected in the community of Flagstaff to investigate the impact of *C. difficile* colonization within an entire geographic region (Stone et al. 2016). The samples were collected from the ground without any connectivity to the participant or epidemiological data acquisition. *C. difficile* was detected in 16.8% (33/197) of the canine faecal samples with 54.5% (18/33) being toxigenic. The authors concluded that dogs may probably serve as a source of infection for human CA-CDI. Nevertheless, studies on *C. difficile* isolation rates from dogs and cats in the community and potential transmission routes within households are very limited.

Coexistence of multiple strains in one faecal sample

The fact that different RTs were isolated in one faecal sample of two study participants is of particular interest. One mixed infection was detected in a human participant harbouring the toxigenic and potentially highly virulent RT 078 simultaneously with the non-toxigenic RT 003, whereas the non-toxigenic *C. difficile* RTs 010 and 039 could be isolated from a dog. The presence of multiple *C. difficile* strains in the same faecal sample has already been described by Eyre et al. (2012) and Shaughnessy et al. (2016). Eyre et al. reported mixed infections in 7.2% (21/292) of human CDI patients in a study focussing on *C. difficile* reinfection rates. Yet, the comparison with the study presented here is biased as only toxigenic *C. difficile* strains were included, molecular typing was performed by MLST, and sample pairs collected within 0 to 7 days were defined as the same case. Additionally, Wroblewski et al. (2009) reported heterogeneous *C. difficile* isolates in 13.0% (3/23) of human faecal specimens. In a recent *C. difficile* prevalence survey on dogs with digestive disorders 13 of 107 (12.1%) dogs were tested positive; different RTs were found in samples of two dogs and in both cases the non-toxigenic RT 010 was involved (Orden et al. 2017). Interestingly, in one of the canine participants two RT 010 isolates showed significantly different antimicrobial susceptibility patterns. The presence of multiple RTs of *C. difficile* was recently reported for vegetables as well, e.g. in 20.8% (5/24) of potatoes (Lim et al. 2018).

Thus, Tanner et al. (2010) raised the question whether characterization of only one suspicious colony per sample was sufficient.
Nevertheless, the impact of colonization with different *C. difficile* strains on the patient is not fully understood, yet, and is probably associated with recurrent CDI (van den Berg et al. 2005). Furthermore, *C. difficile* isolates carrying antimicrobial resistance determinants could transfer those genes horizontally to a naïve co-colonizing strain (Stone et al. 2016). To evaluate the epidemiological significance of co-colonization, and the impact of probable microevolutionary effects, more studies are needed.

Presence, characteristics and impact of toxigenic and non-toxigenic *C. difficile* RTs
In the study presented here, toxigenic *C. difficile* RTs predominated with 61.5% (16/26) and 77.8% (14/18) of the animal and human isolates. Of those, toxigenic isolates encoding for the major toxins A and B as well as for the binary toxin were found in 12.5% (2/16) of animal and 21.4% (3/14) of human *C. difficile* isolates. Those findings were in concordance with the results of a Dutch cross-sectional study investigating the colonization rates in humans within the community; the authors reported that 70.0% (21/30) of the *C. difficile* isolates were toxigenic (Zomer et al. 2017). For dogs, more than 50% (18/33) of *C. difficile* isolates detected within the community have been reported to be toxigenic (Stone et al. 2016). Furthermore, among the toxigenic RTs RT 014/0 and 078 were most frequently isolated; this finding is also congruent with the results presented here. Another Dutch prevalence survey found an overall proportion of 53.1% (51/96) toxigenic *C. difficile* strains in healthy individuals of seven different animal species (Koene et al. 2012). Interestingly, all samples originating from livestock (pigs, cows, and sheep) as well as the majority of the poultry samples harboured toxin-producing strains whereas the pet samples yielded mostly non-toxigenic RTs, with 62.1% (18/29) and 94.4% (17/18) in canine and feline isolates. Although the proportion of toxigenic *C. difficile* isolates clearly contradicts the results presented here, with 50.0% (8/16) and 80.0% (8/10) in dogs and cats, the predominant toxigenic RT isolated from cats and dogs in the study conducted by Koene et al. (2012) was also RT 014/0.

In Europe *C. difficile* diarrhoea in humans is most commonly caused by RT 014/0 strains (Bauer et al. 2011; Janezic et al. 2012). Recently, RT 014/0 has also been isolated as the predominant strain in diarrhoeic dogs sampled in veterinary clinics in Spain (Andrés-Lasheras et al. 2018). Additionally, the findings of Andrés-Lasheras et al. showed that dogs suffering from dysbiosis could harbour non-toxigenic *C. difficile* strains, as well. Although RT 014/0 produces the major toxins A and B it is rarely involved in severe epidemic outbreaks. Yet, this RT seems to comprise particular adaptive capabilities since it can be found in a wide variety of animal species (Janezic et al. 2014; Knight et al. 2017). In the study presented here RT 014/0 was determined in 38.5% (10/26) of animal and 22.2% (4/18) of human isolates, thereby, being also the most prevalent among the 17 different ribotypes found here. Phylogenomics of the RT 014/0 lineage led to the assumption that this RT is of rising importance in the One Health context due to its wide range dissemination and genomic relatedness in animal and human strains (Knight et al. 2015). Moreover, the RT 014/0 lineage has been described to comprise a large open pan-genome with RT 014/0 strains sharing a common evolutionary origin suggesting probable transmission events between human and pigs (Knight et al. 2017). The success of the RT 014/0 lineage is based on the ability to inhabit a wide range of species and a worldwide distribution (Knight et al. 2017; Janezic et al. 2014). The flexibility and diversity of this lineage is also mirrored by the RT 014/0 isolates detected in the study presented here. MST based on MLVA data displayed clonal relationships of human, canine and feline isolates as well as single dispersed isolates with STRD >10.

Furthermore, clonally related RT 014/0 isolates were detected within two independent cat-owning households participating in this study. The strains originating from different households clustered within different clonal complexes and MLVA results revealed that partner cats sharing the same environment harboured clonally related RT 014/0 strains suggesting intra-household transmission events. This is in accordance with the considerable endemic potential of RT 014/0 as described by Janezic et al (2012; 2014). Nevertheless, it remains unclear so far whether a common source of infection or intra- and/or interspecies transmission caused widespread dissemination of RT 014/0.

In contrast to RT 014/0, RT 078 strains produce the binary toxin CDT in addition to toxins A and B, and are often described as potentially highly pathogenic or hypervirulent in humans (Stabler et al. 2012). Reviewing the changing epidemiology of CDI, Freeman et al. (2010) pointed out that the rate of CDI caused by RT 078 has been on the rise in the Netherlands, e.g. from 3% in 2005 to 13% in 2007. Besides, RT 078 is the third most prevalent RT in hospital patients in European countries (Bauer et al. 2011) and has also been described as the most common RT in bovine and porcine populations (Keel et al. 2007; Schneeberg et al. 2013b). Regardless of the overall low isolation rates in this study, RT 078 was also the third most prevalent RT among the study population presented here, relating to small companion animals and their owners. Moreover, RT 078 is known as a livestock-associated lineage which is especially linked to CA-CDI (Knight and Riley 2016; Goorhuis et al. 2008a). RT 078 infected CDI-patients are younger and more often associated with the onset of disease apart from hospital settings compared to patients infected with RT 027 (Freeman et al. 2010). Furthermore, a genetic relatedness between human and livestock RT 078 isolates had been reported (Debast et al. 2009; Bakker et al. 2010; Koene et al. 2012). Indistinguishable RT 078 strains had been isolated from farmers and their pigs in the Netherlands (Knetsch et al. 2014; Keessen et al. 2013). Thus, concerns of a zoonotic potential of *C. difficile* had been triggered particularly with regard to the highly virulent RT 078 (Putsathit et al. 2015; Álvarez-Pérez et al. 2017a). Comparing 247 *C. difficile* RT 078 strains of livestock and human origin Knetsch et al. (2018) concluded that RT 078 has presumably frequently been transmitted between animals and humans. Here, we also detected RT 078 isolates originating from human and canine samples with three isolates forming a clonal cluster in the MST deriving from MLVA analysis. This confirmed the high genetic relatedness between RT 078 strains of small companion animals and those of their owners, suggesting that not just livestock but also companion animals might play a role in interspecies transmission of RT 078.

The detection of RT 027 in the study group presented here is of particular interest in the epidemiological context as this strain is known as an epidemic RT which has caused severe outbreaks of HA-CDI since the early 2000s. Its emergence was linked to an increased incidence and severity of disease caused by *C. difficile*, especially in Europe and North America (Freeman et al. 2010; Rupnik 2007). Interestingly, RT 027 seemed to be rare in Asian and Latin American countries (Putsathit et al. 2015; Monteiro et al. 2014; Silva et al. 2015; Salazar et al. 2017); presumably, this was due to under-detection (Monteiro et al. 2014). Initially, the geographical dissemination seemed to be mainly restricted to industrialized, western countries; this has changed during the last decade. The first cases of RT 027 infections outside of Europe and North America had been reported in Asia in 2005 and in South America in 2009. Two distinct epidemic lineages of this RT had managed a transcontinental spread (Valiente et al. 2014; He et al. 2013). Not only humans but also animals are susceptible to this human epidemic RT. RT 027 has also been isolated from cattle and horses (Janezic et al. 2014), though data for companion animals are rare. There are only two reports on RT 027 in small companion animals describing its isolation from two healthy hospital visitation dogs in Canada (Lefebvre et al. 2006a; Lefebvre and Weese 2009). The

canine isolate detected in this study is the first *C. difficile* RT 027 strain originating from a dog outside of Canada. The dog suffered from symptoms comparable to those in human patients with recurrent episodes of diarrhoea and the anamnesis of previous treatment with antibiotics and PPI. The comparison of whole genome data from the canine and human RT 027 strains will enable to further elucidate the evolution of this highly virulent epidemic strain. The detection of this important human pathogen in a dog proves that RTs primarily associated with human infection do also occur in companion animals. Hence, this raises again the question of the *C. difficile* reservoir and whether animals or rather humans are a source of infection for the counterpart.

Among the non-toxigenic strains RT 010 was the most frequently detected RT in all pet samples collected within this study. Although the toxin-producing strains prevailed within our study population, RT 010 was the most commonly isolated RT after the toxigenic RT 014/0 in canine samples. RT 010 is an important and widely distributed strain which can be isolated from humans and various animal species (Janezic et al. 2014; Terhes et al. 2006). Yet, in this survey the majority of isolated RT 010 was closely related. According to MLVA data they clustered in a genetically related cluster (GRC) together with the newly described RT 010/FLI01. This GRC comprised RTs of human, canine and feline origin displaying a high similarity within the repetitive genome of RT 010, thereby, supporting the zoonotic potential of *C. difficile*. Though non-toxigenic, many antibiotic resistance genes have been identified in RT 010 strains of human and animal origin (Álvarez-Pérez et al. 2015; Janezic et al. 2012; Avberšek et al. 2014) raising assumptions whether those strains could serve as a reservoir for antibiotic resistance determinants (Janezic et al. 2012). Although non-toxigenic *C. difficile* strains do not cause CDAD, they might play a role in distributing antibiotic resistance genes, e.g. via horizontal gene transfer, to toxigenic strains. Furthermore, RT 010 has also been detected in the environment, e.g. in puddle water and soil (Janezic et al. 2016). Those findings indicate an exchange of non-toxigenic *C. difficile* between diverse habitats facilitating transmission, e.g. through animal transports, water recycling or manuring (Janezic et al. 2016). Here, the spore-forming ability of *C. difficile* increases its chance to survive under several conditions, thereby enhancing dissemination. On the other hand, it has been shown that spores of non-toxigenic *C. difficile* successfully protected against colonization or infection with toxigenic *C. difficile* RTs (Keessen and Lipman 2012; Sambol et al. 2002). Non-toxigenic *C. difficile* are presumably part of the microbiota and its overall composition and dynamics are crucial for providing a protective effect against infections (Morgan et al. 2015). Interestingly, Nagaro et al. (2013) described that non-toxigenic strains even had a positive effect in a hamster model when the animals were challenged with potentially highly pathogenic strains of RT 027. Yet, in the study presented here, we detected a mixed infection in a human who harboured the potentially highly pathogenic RT 078 and the non-toxigenic RT 003, simultaneously. This shows that the role of non-toxigenic strains in the pathogenesis of CDI and the coexistence of multiple *C. difficile* strains still needs to be further elucidated. However, the high proportion of toxigenic strains isolated from human and animal participants sharing the same RT in this study raises concerns that interspecies transmission is likely to occur and might have an impact in the One Health context.

Intra-household transmission

In a Canadian retrospective survey, 2222 index cases were screened for CDI-infected household contacts over a 9-year period (Pepin et al. 2012). In total, only eight case-pairs were tracked and the authors reported an increased relative risk of CDI for family members for up to three months. Nonetheless, the absolute numbers indicate only an infrequent and low risk for intrafamilial transmission. Over a 5-year period, index cases were followed up in the UK and 3 intrafamilial case-pairs out of 238 confirmed CDI cases were identified (Baishnab

et al. 2013). Again, the authors concluded that household transmission between family members is possible but infrequent. Nonetheless, the isolated RTs were indistinguishable by PCR-ribotyping and MLVA. However, in both publications no animal contacts within the households were considered. In 2010, Weese et al. investigated 84 Canadian dog-keeping households for the presence of *C. difficile*. The authors detected *C. difficile* from 10.1% (14/139) of dogs and in 31.0% (26/84) of the households with RT 027 being the most common identified strain in the household environment. In four households *C. difficile* was isolated from dogs and their environment, but in all cases the detected RTs differed. It was concluded that exposure to *C. difficile* in the household environment seems to be common and that dogs did presumably not play a significant role as a contaminant. Unfortunately, the animal owners were not part of the study population within this survey.

Within this study *C. difficile* was not detected in dogs or cats and their owners sharing the same household, simultaneously. Yet, the overall low isolation rate within the community will have impaired the chances of detecting isolate pairs. Shaughnessy et al. (2016) and Loo et al. (2016) reported on simultaneous sampling of humans and animals sharing the same household for the first time. Shaughnessy et al. focussed on recurrent CDI-cases. Households with patients suffering from recurrent CDI who underwent faecal microbiota transplantation were included. Among those, eight animal-keeping households were also screened but *C. difficile* could not be isolated from a pet. However, specific household sites such as the vacuum cleaner and the bathroom were described as areas with a high risk for *C. difficile* contamination. Whether persistent spores in the household environment had caused a recurrence of CDI in human participants in this study remained unclear. Loo et al. investigated *C. difficile* transmission from human CDI-index cases to their household contacts involving persons and small companion animals. A total of 51 households participated over a follow-up period of four months in this Canadian prospective study. Among those, 15 pet-owning households participated with cats (9), dogs (5) and one bird. Of those, 26.7% (4/15), two dogs and two cats, were tested positive for toxigenic *C. difficile*; the authors described the animals as asymptomatic carriers. Interestingly, PFGE analysis revealed that the *C. difficile* isolates from dogs and cats as well as from human patients living in the same household were indistinguishable or closely related. Therefore, the authors concluded that transmission between human CDI patients and their dog or cat is likely to occur. Although the authors presented indistinguishable *C. difficile*-PFGE-profiles originating from animal-human isolate pairs, the discriminatory power of PFGE does not enable a full molecular characterization of strains. Hence, zoonotic transmission of *C. difficile* remains to be verified. Although the study populations described by Shaughnessy et al. (2016) and Loo et al. (2016) consisted of human participants with a history of CDI in contrast to the participants in the study presented here, we confirmed that clinically relevant RTs are shared between mainly asymptomatic human carriers and small companion animals. Moreover, the high proportion of toxigenic strains raises concerns that interspecies transmission would have a clinical impact. Additionally the overlap in human and animal RTs, which has also been described before and the genetic relatedness of certain strains suggests that interspecies transmission as well as zoonotic transmission is probably possible to occur (Rabold et al. 2018).

6.3. Epidemiological factors associated with *C. difficile* isolation in dogs, cats and their owners

The first risk factors for colonization resp. development of CDAD in dogs and cats had been described by Clooten et al. (2008) in a prospective study. The authors stated that antimicrobial therapy prior hospital stays and intake of immunosuppressive drugs during hospitalization are independently associated with the development of hospital-acquired *C. difficile* diarrhoea. On the contrary, there was no significant association between faecal shedding of toxigenic strains

at the time of hospital admission and diarrhoea in small companion animals. The authors assumed that asymptomatic colonization with non-toxigenic or toxigenic *C. difficile* might protect against CDAD, which had already been discussed for humans.

Statistical analysis of the epidemiological data acquired within this study revealed that common risk factors associated with human CDI, such as age and antibiotic treatment, were also associated with CITP in dogs and cats. The univariate model indicated that dogs and cats aged between five to nine years had a significantly higher chance for *C. difficile*-positivity. Similarly, dogs from the age of seven years or even older had a higher chance to be positively tested for the presence of *C. difficile* in a survey studying small companion animals in veterinary hospital settings (Álvarez-Pérez et al. 2017b). In contrast, human participants younger than 12 months of age of the study population presented here had the highest risk to be affected by *C. difficile*-CITP. This is in accordance with the results of a recent prospective surveillance study on household contacts of CDI-patients where children had the highest chance for asymptomatic colonization (Loo et al. 2016). Since the first description of *C. difficile* in 1935, high isolation rates in young children have regularly been reported (Hall 1935; Lees et al. 2016). Still, the clinical impact of *C. difficile* colonization in children remains uncertain (Enoch et al. 2011). Nonetheless, infants being ≤3 years old had the highest risk of infection in a US-surveillance study on CDI in children (Wendt et al. 2014).

Apart from age the treatment with antibiotics has been recognized as a major risk factor for CDI for a long time (Freeman and Wilcox 1999). It is beyond doubt that antibiotics have a disrupting effect on the healthy gut microbiota and, thereby, enables *C. difficile* to cause disease. This was also confirmed in a multivariate model for animal and human participants in this study with a significant 4- resp. 9-fold increase of *C. difficile*-positivity. The impact of treatment with PPI or AID as a risk for human CDI has previously been discussed controversially (Suissa et al. 2012; Lowe et al. 2006; Dial et al. 2004; Chen et al. 2016). In the study presented here, the intake of PPI or AID was not significantly associated with the isolation of *C. difficile* in animal owners. Yet, in small companion animals the intake of PPIs was significantly linked to *C. difficile*-CITP. To the current knowledge, this is the first report describing the association between PPI treatment and *C. difficile*-positivity in dogs and cats. Similar to human therapy, the use of PPI in animals aims to suppress gastric acid production. Within the group of participating animals, dogs had been more often medicated with PPI than cats (9/10), suggesting that PPI-treatment in dogs severely increases the chances for faecal shedding of *C. difficile*. Inappetence was also linked to *C. difficile*-positivity in the univariate analysis of this study. Nevertheless, it remains speculative whether inappetence can lead to a disruption of the gut microbiota thereby enabling colonization, or whether infection with *C. difficile* CDI causes inappetence. Small companion animals were six-fold more likely tested positive for *C. difficile* when they were suffering from inappetence or an acute disease in the univariate model. However, antibiotic treatment in animals with an acute disease may bias potential risk factors.

Despite an impressively high number of surveys reporting on high isolation rates in a variety of food products of animal and plant origin, the intake of specific food products is not significantly associated with *C. difficile*-positivity in human participants of this study. The role of food-products in the epidemiology of *C. difficile* infections may have been over-estimated, since. Cross-contamination during food processing, especially in the retail meat industry, and even in laboratories is likely to occur and may have caused high isolation rates (Weese 2010; Marsh 2013; Marsh et al. 2011). Moreover, although some surveys report on the detection of human pathogenic RTs in different food products, mainly of animal origin, the role of *C. difficile* as a foodborne or feedborne pathogen could not be proved until now (de

Boer et al. 2011). Although ingestion of low quantities of *C. difficile* through contaminated food, presumably, occurs regularly (Weese et al. 2010b), it is possibly not the main source of infection for humans and animals. Nonetheless, ongoing research is required to elucidate the epidemiological role of contaminated food particularly in CA-CDI (Weese et al. 2010b; Lim et al. 2018).

Unexpectedly, animals fed with dry feed had a significantly decreased likelihood for *C. difficile*-CITP. Feeding on dry feedstuffs also remained as an independent factor in the final multivariate model. Hence, the chance for dogs or cats being tested positive for *C. difficile* decreased approximately 10-times when they were fed with dry feed. Whether the common *ad libitum* feeding of dry feedstuffs in small companion animals has a positive impact on the gut microbiota or whether this is due to the presumably low contamination of dry feedstuffs still has to be unraveled. High temperatures applied during desiccation of feed processing could explain low contamination. Although *C. difficile* spores are able to withstand temperatures of up to 71°C over a period of 120 min (Rodriguez-Palacios et al. 2007), survival of *C. difficile* during industrialized processing of commercial animal feed seems highly unlikely since extrusion processes with temperatures up to 200°C are applied (van Rooijen et al. 2014; Tran et al. 2008). The combination of feeding strategy and a probably low contamination of dry feedstuffs seems to have a positive effect on the intestinal microbiota of companion animals.

In accordance with well-known risk factors for human CDI, animal owners not working in agriculture, food production or in the health care sector had a significantly 5-fold decreased likelihood for *C. difficile*-positivity in the univariate analysis. Nonetheless, working in agriculture, the food production sector or in health care were no significant risk factors. A possible explanation is the negligible absolute number of participants working in those fields which probably impairs statistical power. However, participants with no current occupation had a higher chance of *C. difficile*-CITP. This was a surprising finding, which was hard to explain. Nevertheless, the multivariate analysis did not confirm unemployment as an independent variable for our study population.

In spite of the absence of animal-human isolate pairs, the results of the epidemiological analysis of factors associated with *C. difficile*-CITP in our univariate analysis support the hypothesis of a zoonotic potential for *C. difficile.* Specific variables that refer to interactions between animals and humans were significantly associated with the detection of *C. difficile*. In detail, the likelihood for dogs or cats of being tested positive for *C. difficile* tended to increase if the owner suffered from a chronic disease ($p = 0.006$) or the animal was in contact with a diarrhoeic person ($p = 0.022$). This indicates that sharing the environment with humans influences whether companion animals harbour *C. difficile*. This finding supports the results of previous studies describing hospital visitation dogs acquiring *C. difficile* in health-care facilities (Lefebvre et al. 2006a; Lefebvre and Weese 2009). Furthermore, regular contact to immunocompromised persons as a significant risk factor has already been reported (Weese et al. 2010a). Even in our multivariate model, contact to a person with diarrhoea was found to be an independent risk factor, increasing the chances for dogs and cats for *C. difficile*-CITP three-fold. Therefore, the impact of animals for human CDI probably requires reconsideration as humans might rather pose a risk for animals than vice versa. Nonetheless, they might be a source for reinfection (Rabold et al. 2018).

The study presented here has a few limitations; one being that sampling was restricted to just one faecal sample per individual participant. On the one hand, repeated sampling could have increased detection rates of *C. difficile* as it probably would have identified intermittent and

transmitted shedding. Additionally, the chances to find animal-human isolate pairs would have possibly been increased. On the other hand, however, repeated sampling as an inclusion criterion for valid study participation would have led to considerably reduced numbers of participants. In particular, repeated sampling would have excluded interested animal owners living in households with more than two animals, especially with cats, being confronted with extraordinarily logistic challenge. Another limitation of the study design was that the anamnestic character of the questionnaire did not request the clinical picture of the participant at the time of sampling. The participant was only questioned about a time period preceding study participation. Though, this additional information would have enabled to draw conclusions about the probable correlation between *C. difficile*-positivity and clinical symptoms such as diarrhoea or abdominal pain.

7. Summary

The objective of the study "Occurrence and characterization of *Clostridioides difficile* in small companion animals and their owners" was to assess the significance of *Clostridioides* (*C.*) *difficile* within the community with regard to its possible zoonotic impact. Therefore, 1,447 faecal samples were collected to determine the occurrence of *C. difficile* in small companion animals (dogs and cats) and their owners in the first large-scale Germany-wide survey. PCR ribotyping, Multilocus VNTR Analysis (MLVA) and PCR detection of toxin genes were used to characterize isolated *C. difficile* strains. Thereby, the role of animals carrying human pathogenic strains was to be explained unravelling whether animals serve as an infectious source for human *C. difficile* infections (CDI). Additionally, a database was defined and logistic regression used to identify putative factors associated with faecal shedding of *C. difficile* in humans and companion animals to further elucidate epidemiological dynamics of CDI.

The isolation rates of *C. difficile* in German dogs, cats and their owners were quite similar and low (between 2.5 and 3.0%). Isolate pairs from humans and animals sharing the same household could not be detected. However, identical RTs were isolated from partner cats in two independent households; this indicates that intra-species transmission or at least acquisition of *C. difficile* from the same source is possible. Additionally, well known human RTs like RT 010, the hospital-associated lineage RT 014/0, and the highly virulent RTs 027 and 078 also occur in small companion animals suggesting at least a common source of infection. Furthermore, the canine RT 027 isolate is of particular interest since this is the first report on this RT in a dog outside Canada.

The analysis of factors associated with *C. difficile*-positivity showed that already previously defined risk factors for *C. difficile* colonization or infection in humans also apply in companion animals. Dogs and cats were at a higher risk for faecal shedding of *C. difficile* when they were middle-aged or older, suffered from inappetence or acute disease, were regularly treated with proton pump inhibitors or had been medicated with antibiotics within the last three months prior study participation. Moreover, the contact to a person with diarrhoea and sharing the household with an animal owner who is chronically sick significantly increased the chances for small companion animals to be affected by *C. difficile*. Anyhow, the finding that the consumption of dry feedstuffs had a protective effect on dogs or cats with regard to *C. difficile*-positivity was surprising.

Although the source of infection for CDI could not be defined and intake of certain food was not significantly associated within the study population examined here, single variables indicate an impact of interaction between animals and humans on the *C. difficile* epidemiology. Interestingly, despite the assumption that animals might serve as an infectious source for human CDI, the results presented here may also suggest the opposite. Even though a definite zoonotic link was not found it cannot be excluded either. Therefore, further studies involving human and animal participants are necessary to define possible sources of *C. difficile* acquisition and to prove its zoonotic character. In conclusion, the results described in this study were suitable to gain more insights into the epidemiology of the community-associated occurrence of *C. difficile* in small companion animals and their owners.

8. Zusammenfassung

"Vorkommen und Charakterisierung von *Clostridioides difficile* bei kleinen Haustieren und ihren Besitzern"

Das Ziel der vorliegenden Studie "Vorkommen und Charakterisierung von *Clostridioides difficile* bei kleinen Haustieren und ihren Besitzern" war, das Auftreten von *Clostridioides* (*C.*) *difficile* außerhalb von Einrichtungen der Gesundheitswesen im Hinblick auf eine mögliche zoonotische Bedeutung des Erregers zu untersuchen. Dazu wurden 1.447 Stuhlproben in der ersten großangelegten Deutschland-weiten Studie gesammelt, um das Vorhandensein von *C. difficile* bei Tierbesitzern und ihren Heimtieren (Hunden und Katzen) zu ermitteln. Die isolierten *C. difficile*-Stämme wurden mithilfe von PCR Ribotypisierung, Multilocus VNTR Analyse (MLVA) und PCRen zum Nachweis von Toxingenen charakterisiert. Dabei sollte auch die Bedeutung von Tieren, die humanpathogene Stämme tragen, in Hinblick auf die Frage, ob diese Tiere eine Infektionsquelle für humane Infektionen sein könnten, beurteilt werden. Um die epidemiologische Dynamik besser zu verstehen, wurden mögliche Faktoren, die mit der Ausscheidung von *C. difficile* bei Haustieren und ihren Besitzern assoziiert sind, statistisch evaluiert.

Die Isolationsraten von *C. difficile* bei deutschen Heimtieren und ihren Besitzern waren relativ ähnlich und gering (zwischen 2,5 and 3,0%). Isolate-Paare von Menschen und Tieren, die im selben Haushalt leben, konnten nicht gefunden werden. Jedoch wurden identische Ribotypen von Partnerkatzen in zwei unabhängigen Haushalten isoliert, was für eine Übertragung innerhalb eines Haushalts oder zumindest für die Aufnahme von *C. difficile* aus derselben Infektionsquelle spricht. Darüber hinaus konnte nachgewiesen werden, dass beim Menschen häufig vorkommende Ribotypen wie RT 010, die Krankenhaus-assoziierte Linie RT 014/0 und die hochvirulenten Ribotypen 027 und 078 auch bei Hunden und Katzen vorkommen, was für einen epidemiologischen Zusammenhang und die Möglichkeit einer zoonotischen Übertragung spricht. Desweiteren ist die erste Beschreibung eines caninen RT 027-Stammes außerhalb Kanadas von besonderem Interesse.

In der Analyse von Faktoren, die mit dem Nachweis von *C. difficile* assoziiert sind, konnte gezeigt werden, dass bereits beschriebene Risikofaktoren für die Kolonisation oder Infektion mit *C. difficile* beim Menschen auch für Heimtiere zutreffen. Hunde und Katzen hatten ein erhöhtes Risiko positiv auf *C. difficile* getestet zu werden, wenn sie mittleren Alters oder älter waren, an Inappetenz oder einer akuten Krankheit litten, regelmäßig Protonpumpenhemmer einnahmen oder innerhalb der letzten drei Monate vor Studienteilnahme antibiotisch behandelt worden waren. Außerdem verstärkte der Kontakt zu einem Menschen mit Durchfallsymptomatik oder das Zusammenleben mit einem chronisch kranken Tierbesitzer signifikant die Wahrscheinlichkeit für Heimtiere von *C. difficile* betroffen zu sein. Überraschend war das Ergebnis, dass die Aufnahme von Trockenfutter einen schützenden Effekt für Hunde und Katzen hatte.

Obwohl die Quelle für Infektionen mit *C. difficile* nicht definiert werden konnte und auch die Aufnahme bestimmter Lebens- bzw. Futtermittel nachweislich keine Signifikanz in der hier untersuchten Studienpopulation aufwies, deuteten einzelne Variablen auf einen Einfluss der Interaktion zwischen Tieren und Menschen in der *C. difficile*-Epidemiologie hin. Interessanterweise lassen die hier erzielten Ergebnisse entgegen der vorherrschenden Annahme, dass Tiere als Infektionsquelle für humane *C. difficile*-Infektionen dienen könnten, auch eine mögliche Übertragung von Mensch zu Tier vermuten. Es konnte zwar keine zoonotische Erregerübertragung gefunden werden, jedoch kann diese auch nicht ausgeschlossen werden. Deshalb ist es notwendig, dass weitere Studien humane und tierische

Studienteilnehmer involvieren, um mögliche Infektionsquellen für *C. difficile*-Erkrankungen zu erforschen und das zoonotische Potenzial zu evaluieren. Schließlich lässt sich zusammenfassen, dass die beschriebenen Studienergebnisse unterstützend dazu beitragen, epidemiologische Zusammenhänge des Vorkommens von *C. difficile* in der Gesellschaft bei Heimtieren und ihren Besitzern besser zu verstehen.

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10. Appendix

The Appendix depicts the developed material used for participant recruitment, instruction and questionning in the following order: (1) Flyer, (2) Instruction sheet for sample handling and posting ("Hinweise zum Probenversand"), (3) Information sheet about study design ("Information für Studienteilnehmer/innen zum Forschungsvorhaben 'Untersuchung zur Prävalenz und Typisierung von *Clostridium difficile* bei Haustieren und ihren Haltern'"), (4) Declaration of consent for human participants ("Einverständniserklärung des Tierhalters/der Tierhalterin"), (5) Declaration of surrender for animal samples ("Überlassungserklärung des Tierhalters/der Tierhalterin"), and (6) Questionnaire.

Sehr geehrte Tierhalterin, sehr geehrter Tierhalter!

Die **Freie Universität Berlin** führt gemeinsam mit dem **Friedrich-Loeffler-Institut** und dem **Robert Koch-Institut**, unterstützt durch die **Nationale Forschungsplattform für Zoonosen** und gefördert durch das **Bundesministerium für Bildung und Forschung** eine Studie zur Häufigkeit und Charakterisierung des Durchfallerregers ***Clostridium difficile*** bei **Hunden, Katzen** und **ihren Besitzern** durch.

Dafür benötigen wir Ihre Unterstützung!

Wenn wir Ihr Interesse geweckt haben, sprechen Sie uns bitte an.

Werden Sie aktiver Teil einer wichtigen Studie über Mensch und Tier.

Sie können uns auch per E-Mail kontaktieren.

KONTAKT UND INFORMATION

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Teilnehmer

für eine bundesweite Studie

gesucht

„Untersuchung zum Vorkommen und Typisierung von *Clostridium difficile* bei Hunden und Katzen sowie ihren Besitzern“

Freie Universität Berlin

Institut für Mikrobiologie und Tierseuchen
FU Berlin, Fachbereich Veterinärmedizin
Dr. Antina Lübke-Becker

FRIEDRICH-LOEFFLER-INSTITUT
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Institut für bakterielle Infektionen und Zoonosen
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UNTERSTÜTZT DURCH
Nationale Forschungsplattform für Zoonosen

GEFÖRDERT VOM
Bundesministerium für Bildung und Forschung

CLOSTRIDIUM DIFFICILE

Clostridium difficile (C. difficile) **ist ein wichtiger Krankenhauskeim und zunehmend treten Stämme dieses Bakteriums auf, die resistent gegen Antibiotika sind.**

***C.-difficile*-Bakterien können sowohl den Menschen als auch Tiere infizieren.**

Beim Menschen kann die Infektion mit *C. difficile* zu unterschiedlich schwer verlaufenden Erkrankungen des Darmes führen, Symptome reichen von leichtem Durchfall bis zu einer ausgeprägten Entzündung des Darmes, die einen chirurgischen Eingriff erfordert. Besonders ältere Menschen mit Begleiterkrankungen und vorausgegangener Einnahme von Antibiotika können schwer erkranken. Der Erreger kann gelegentlich auch bei vollkommen gesunden Menschen gefunden werden und hat dann in der Regel keine klinische Bedeutung. *C. difficile* wird in diesem Fall durch die normale Darmflora kontrolliert, nur wenn die normale Darmflora geschädigt wird, z.B. durch die Einnahme von Antibiotika, kann es zur Erkrankung kommen. Allerdings ist nicht jeder Durchfall während einer Antibiotikaeinnahme durch *C. difficile* verursacht.

Die medizinische Bedeutung des Nachweises von *C. difficile* bei Tieren ist noch ungeklärt. Wir wissen jedoch, dass es auch bei Tieren zu Durchfall-Erkrankungen kommen kann. Es ist derzeit unklar, ob die Bakterienstämme, die bei Tieren zu finden sind, auch eine Gefahr für den Menschen darstellen.

ZWECK DER STUDIE

Wir hoffen herauszufinden, ob das Bakterium *C. difficile* vom Tier auf den Menschen oder auch vom Menschen aufs Tier übertragen werden kann.

Unser Ziel ist es, statistisch auswertbare Daten zu erhalten, eine Einschätzung zu Übertragungswegen und ggf. Empfehlungen zur Prävention für Tierhalter und Tierkliniken aussprechen zu können.

TEILNAHME

Die Studie besteht aus 2 Teilen: Teilnehmer/innen werden gebeten, einen Fragebogen für sich und ihr Tier auszufüllen. Außerdem erhalten Mensch und Tier eine Laboruntersuchung ihrer Stuhlproben auf *C. difficile*.

Die Teilnahme an der Studie ist kostenlos.

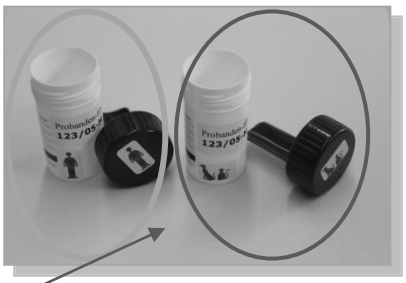
Hinweise zum Probenversand

Sie haben heute folgende Untersuchungsmaterialien zur Durchführung der Studie erhalten. Wir bitten Sie, die folgenden

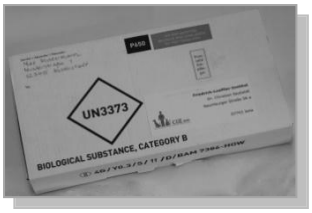
Wir empfehlen Ihnen, die beigelegten Laborhandschuhe zum Gewinn der Stuhlproben zu verwenden.

Probenentnahme Tierhalter/in
Bitte verwenden Sie zur Entnahme Ihrer Stuhlprobe den beigelegten Stuhlfänger und befolgen die Hinweise der Packungsbeilage.

Bitte befüllen Sie das mit dem Menschen und der grünen Probanden-ID gekennzeichnete Probenröhrchen mindestens bis zur Hälfte mit der gewonnenen Probe.



Probenentnahme Tier
Falls möglich gewinnen Sie die Stuhlprobe Ihres Tieres bitte zeitnah nach dem Stuhlgang. Bitte achten Sie darauf, dass der Stuhl nach Möglichkeit frei von Verunreinigungen ist (z.B. Katzenstreu, Gras, Sand o.ä.). Befüllen Sie das mit den Tieren und der violetten Probanden-ID gekennzeichnete Probenröhrchen ebenfalls mindestens bis zur Hälfte.



Versand der Proben

1. Gut verschlossene Probenröhrchen in den hellblauen Schutzumschlag
2. Verschlossener Schutzumschlag in die vorbereitete Versandbox
3. Verschließen und schicken Sie die Versandbox an die angegebene Adresse. Sie brauchen die Box nicht zu frankieren, das Porto zahlt der Empfänger.

Herzlichen Dank!

Information für Studienteilnehmer/innen zum Forschungsvorhaben

„Untersuchung zur Prävalenz und Typisierung von *Clostridium difficile* bei Haustieren und ihren Haltern“

Sehr geehrte Studienteilnehmerin,
sehr geehrter Studienteilnehmer,

Sie sind heute gefragt worden, ob Sie an einem **Forschungsprojekt zu Vorkommen und Typisierung von *Clostridium difficile* und der Sammlung der dazugehörigen Daten für epidemiologische Forschungszwecke** teilnehmen würden/möchten.

Einleitung

Clostridien sind Bakterien, die Sporen bilden können und bevorzugt in anaeroben, d.h. sauerstoffarmem Milieu wachsen. Die Infektion mit *Clostridium difficile* erfolgt oral (Aufnahme über den Mund) und kann zu Erkrankungen des Darmes, die mit Durchfall einhergehen können, führen. Aus der Gruppe der anaeroben Bakterien verursacht *Clostridium difficile* die häufigsten Krankenhausinfektionen. In Nordamerika und Europa werden vermehrt Erkrankungen durch diesen Erreger festgestellt. Zunehmend treten Varianten dieses Bakteriums auf, die resistent gegen Antibiotika sind. *Clostridium difficile*-Bakterien infizieren sowohl den Menschen als auch Tiere. Es ist derzeit unklar, ob die Bakterienstämme, die in Tieren zu finden sind, auch eine Gefahr für den Menschen darstellen.

Zweck der Studie

Ziel dieses Forschungsprojektes ist es, das Vorkommen von *Clostridium difficile* bei Mensch und Tier sowie die Eigenschaften der jeweiligen Erreger zu charakterisieren. Wir werden die Eigenschaften der Bakterienstämme des Tieres mit denen des Menschen vergleichen. Wir erhoffen uns, Einschätzungen zu Übertragungswegen und ggf. Empfehlungen zur Prävention für Tierhalter und eventuell Tierkliniken aussprechen zu können. Dafür werden in dieser Studie Proben von Haustieren und ihren Besitzern gesammelt. Die ausführenden Institutionen sind das Institut für Mikrobiologie und Tierseuchen der Freien Universität Berlin, das Friedrich-Loeffler-Institut und das Robert Koch-Institut.

In dieser Studie sollen folgende Untersuchungen durchgeführt werden:

1. Ihre Stuhlprobe soll im Friedrich-Loeffler-Institut in Jena auf das Vorhandensein des Erregers *Clostridium difficile* untersucht werden. Dort sollen auch Untersuchungen zu den Eigenschaften der isolierten Bakterienstämme durchgeführt und Ihre Proben tiefgefroren für eventuell weitere Untersuchungen gelagert werden.

2. Mit einem Fragebogen sollen Informationen zum allgemeinen Gesundheitszustand und möglichen Vorerkrankungen, zu Tierkontakten sowie allgemeinen Lebensumständen gesammelt werden. Diese Daten werden durch das Robert Koch-Institut untersucht. Der Fragebogen und die Stuhlprobe erhalten einen Teilnehmer-Code, der die eindeutige Zuordnung der erhobenen Daten zu den ggf. aus dem Stuhl isolierten Bakterien ermöglicht. Ihre persönlichen Daten (Name und Anschrift) verbleiben bei uns und werden nach einer Aufbewahrungsdauer von 10 Jahren gelöscht. Rückschlüsse des Robert Koch-Instituts, des Friedrich-Loeffler-Instituts oder von Dritten auf Ihre Person sind damit ausgeschlossen.

Durch die Analyse der in dieser Datenbank gesammelten Informationen möchten wir mögliche Beziehungen zwischen den Bakterieneigenschaften und dem Infektions- und

Krankheitsgeschehen erkennen und ggf. daraus Maßnahmen zur besseren Vorbeugung von Infektionen mit *Clostridium difficile* ableiten.

3. Am Friedrich-Loeffler-Institut soll eine Daten- und Biomaterialbank (Stuhlproben) etabliert werden, die langfristig betrieben wird, um das Infektionsgeschehen in Deutschland über längere Zeit zu beurteilen. Das bedeutet, dass Daten in elektronischer Form gespeichert und die organischen Proben tiefgefroren gelagert werden. Diese Daten- und Probensammlung soll für andere Studienaspekte nutzbar sein. Der Zugang für Forschungsarbeiten anderer auf dem Gebiet der Infektionskrankheiten arbeitenden wissenschaftlichen Einrichtungen wird nur gewährt, wenn die hier beschriebenen Regeln zum Schutz Ihrer Daten und Rechte verpflichtend eingehalten werden.

Was bedeutet die Studienteilnahme für Sie?

Sie helfen durch Ihre Teilnahme mit, neue Erkenntnisse zum Vorkommen von *Clostridium difficile* und Risikofaktoren einer Trägerschaft oder Erkrankung zu erlangen. Zusätzlich werden Sie als Studienteilnehmer/in über die Ergebnisse Ihrer Stuhlprobe innerhalb von 9 Monaten schriftlich durch die Studienverantwortlichen informiert.

Bitte fragen Sie, wenn Sie etwas nicht verstehen oder wenn Sie zusätzlich etwas wissen möchten. Wir werden mit Ihnen auch direkt über die Studie sprechen.

Bei späteren Rückfragen wenden Sie sich bitte auch jederzeit an:

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Bundesministerium für Bildung und Forschung

EINWILLIGUNGSERKLÄRUNG DES TIERHALTERS / DER TIERHALTERIN

Aufkleber
Person ID

Diese Einwilligungserklärung betrifft nur
die Teilnahme des Tierhalters / der Tierhalterin.

Hiermit erkläre ich, _____
Vorname, Name

geb. _____,
Geburtsdatum des/der Studienteilnehmers/in

Adresse _____

_____
Kontaktadresse des/der Studienteilnehmers/in

Telefon _____,

dass ich durch Herrn/Frau _____
Mitarbeiter/Vertretungsberechtigter der Forschungsgruppe

über das Wesen, die Bedeutung, Tragweite und Risiken der wissenschaftlichen Untersuchung im Rahmen der o.g. Studie informiert wurde und ausreichend Gelegenheit hatte, meine Fragen hierzu zu klären. Ich habe insbesondere die mir vorgelegte Teilnehmerinformation verstanden und eine Ausfertigung derselben und diese Einwilligungserklärung erhalten.
Ich bin bereit, an der wissenschaftlichen Untersuchung im Rahmen der o.g. Studie teilzunehmen. Ich übereigne die mir entnommene Stuhlprobe hiermit an die das Forschungsprojekt ausführenden Institutionen. Dabei bin ich mir bewusst, dass dies meine nachfolgend abgegebenen Erklärungen hinsichtlich meines Persönlichkeitsrechts nicht einschränkt.

Ich bin damit einverstanden, dass meine Stuhlprobe gegebenenfalls in weiteren Studien verwendet wird, die der Aufklärung der Epidemiologie von Infektionen durch Darmbakterien dienen.

□ Ja

□ Nein

Ich bin damit einverstanden, dass ich in einem solchen Fall keine individuellen Rückinformationen über die getätigte Forschung erhalte, und ich bin mir bewusst, dass ich für die Überlassung meiner Stuhlprobe keine finanzielle Entschädigung erhalte.

Aufklärung über den Datenschutz

Die Belange der ärztlichen Schweigepflicht und des Datenschutzes werden voll gewahrt.

Durch Ihre Unterschrift auf der Einwilligungserklärung erklären Sie sich damit einverstanden, dass Ihre personenbezogenen Daten zum Zweck der o.g. Studie im Rahmen dieses Forschungsvorhabens erhoben und verarbeitet werden dürfen. Personenbezogene Daten sind z.B. Ihr Name, Geburtsdatum, Ihre Adresse und Daten zu Ihrer Gesundheit oder Erkrankung oder andere persönliche Daten, die während Ihrer Teilnahme an der Studie zweckgebunden erhoben wurden.

Ihre im Rahmen dieser Studie per Fragebogen erhobenen Daten werden mit einer Codenummer versehen, die nur den Studienverantwortlichen der FU Berlin eine Zuordnung dieser Daten zu ihrer Person ermöglicht. Die Daten werden ausschließlich in dieser codierten Form für Zwecke der Forschung und der statistischen Auswertung verwendet. Die personenbezogenen Daten, die Sie identifizieren, werden bei der Projektleiterin gespeichert, weil wir Ihnen das Ergebnis der Laboruntersuchung mitteilen möchten.

Bitte beachten Sie, dass die Ergebnisse der Studie in anonymisierter Form in der medizinischen Fachliteratur veröffentlicht werden können.

Die von Ihnen im Rahmen der o.g. Studie abgegebene Stuhlprobe wird ebenfalls mit der Codenummer versehen und unter dieser Nummer an der FU Berlin, dem Friedrich-Loeffler-Institut und dem Robert Koch-Institut bearbeitet. Rückschlüsse der Mitarbeiter des Friedrich-Loeffler- Instituts und des Robert Koch-Instituts oder von Dritten auf Ihre Person sind damit ausgeschlossen. Nach einem Zeitraum von 10 Jahren werden Ihre personenbezogenen Daten bei uns gelöscht.

Sie haben das Recht, Auskunft über die Sie betreffenden aufgezeichneten Angaben und die Ergebnisse Ihrer Untersuchung zu verlangen. Sie können bei unrichtiger Aufzeichnung von Angaben, die Ihre Person betreffen, auch eine Berichtigung dieser Angaben verlangen. In diesen Fällen wenden Sie sich bitte an die Projektleiterin Frau Dr. A. Lübke-Becker, Tel.: 030-2093-6004, E-Mail: luebke.antina@vetmed.fu-berlin.de.

Sollten Sie einer Weiterverarbeitung Ihrer Daten widersprechen, werden keine weiteren Daten über Ihre Person zum Zweck der o.g. Studie erhoben und aufgezeichnet. Die bis zu diesem Zeitpunkt vorhandenen Daten müssen aber möglicherweise aus Gründen der Sicherheit anderer Studienteilnehmer/-innen und der Wahrung gesetzlicher Dokumentationspflichten weiter verarbeitet werden. Gleiches gilt für eine von Ihnen verlangte Löschung der Sie betreffenden Angaben.

Erklärung zum Datenschutz:

Ich erkläre mich einverstanden, dass die im Rahmen dieser Studie über mich erhobenen Daten/Angaben verschlüsselt auf elektronischen Datenträgern aufgezeichnet und verarbeitet werden sowie als anonymisierte Studienergebnisse veröffentlicht werden können. Auch erkläre ich mich einverstanden, dass meine vorgenannten Daten zum Zweck der oben genannten Studie an das Robert Koch-Institut, Berlin, bzw. das Friedrich-Loeffler-Institut, Jena, übermittelt werden dürfen. Darüber hinaus bin ich mit der Verschlüsselung und Untersuchung meiner im Rahmen dieser Studie entnommenen Stuhlprobe für den Zweck der Studie einverstanden.

Widerruf der Zustimmung zur Datenverwendung:

Ich weiß, dass ich meine Einwilligung jederzeit ohne Angabe von Gründen und ohne nachteilige Folgen für mich gegenüber der einleitend genannten Institution widerrufen kann.

_____

Ort, Datum, Unterschrift Studienteilnehmer/in

Hiermit erkläre ich, den/die o.g. Teilnehmer/in über Wesen, Bedeutung und Risiken der o.g. Studie mündlich und schriftlich aufgeklärt und ihm/ihr eine Ausfertigung der Information sowie dieser Einwilligungserklärung übergeben zu haben.

_____

Ort, Datum, Unterschrift Studienteilnehmer/in

Aufkleber
Tier ID

ÜBERLASSUNGSERKLÄRUNG DES TIERHALTERS / DER TIERHALTERIN

Ich übereigne die meinem Tier entnommene Kotprobe hiermit an die das Forschungsprojekt ausführenden Institutionen.

_____

Ort, Datum, Unterschrift Studienteilnehmer/in

Untersuchungen zur Prävalenz und Typisierung von *Clostridium difficile* bei Haustieren und ihren Haltern

I. Angaben zur Tierhaltung allgemein

1. Halten Sie außer dem heute getesteten Tier weitere Nutz- oder Haustiere?
   ☐ Ja ☐ Nein ☐ Keine Angabe
   ↳ falls ja, welche Tiere (Mehrfachnennungen möglich)? Bitte geben Sie die Anzahl an: .....
   ____ Hunde ____ Katzen ____ Schafe ____ Geflügel ____ Wildtiere ____ Kleinsäuger
   ____ Pferde ____ Rinder ____ Schweine ____ andere (Tierart bitte angeben): .....

2. Hatten Sie in den letzten 12 Monaten mehrfach Kontakt zu anderen Tieren, deren Besitzer nicht Sie sind? (z.B. Haustiere, Jagd...)?
   ☐ Ja ☐ Nein ☐ Keine Angabe
   ↳ falls ja, welche Tiere (Mehrfachnennungen möglich)
   ☐ Hunde ☐ Katzen ☐ Schafe ☐ Geflügel ☐ Wildtiere ☐ Kleinsäuger
   ☐ Pferde ☐ Rinder ☐ Schweine ☐ andere (bitte angeben): .....

Aufkleber Tier ID

II. Angaben zum getesteten Tier

3. Bei dem getesteten Tier handelt es sich um: ☐ Hund ☐ Katze

4. Rasse des Tieres?	5. Geschlecht des Tieres?	6. Kastriert?	7. Alter:
.....	☐ weiblich ☐ männlich	☐ Ja ☐ Nein	..... Jahre

8a) *(falls Hund)* Wie wird dieser Hund hauptsächlich (mehr als die Hälfte der Zeit) gehalten?
☐ im Haus/in der Wohnung
☐ im Zwinger/Garten
☐ im separaten Nebengebäude (z.B. Stall)

8b) *(falls Katze)* Wie wird diese Katze gehalten?
☐ im Haus/in der Wohnung
☐ Freigänger
↳ *falls Freigänger,* wie häufig befindet sich Ihre Katze im Freien?
☐ täglich: ..... Stunden pro Tag.
☐ mehrmals in der Woche: ..... Stunden pro Woche

9. Hat Ihr Tier regelmäßigen Kontakt zu anderen Nutz- oder Haustieren?
   ☐ Ja ☐ Nein ☐ Keine Angabe
   ↳ *falls ja,* zu welchen Tieren (Mehrfachnennungen möglich)?
   ☐ Hunde ☐ Katzen ☐ Schafe ☐ Geflügel ☐ Wildtiere ☐ Kleinsäuger
   ☐ Pferde ☐ Rinder ☐ Schweine ☐ andere (bitte angeben): .....

10. Hatte Ihr Tier in den letzten 12 Monaten regelmäßigen Kontakt zu Jungtieren (z.B. Welpen, Ferkeln, ...)?
    ☐ Ja ☐ Nein ☐ Keine Angabe
    ↳ *falls ja,* zu welchen Tieren (Mehrfachnennungen möglich)?
    ☐ Hunde ☐ Katzen ☐ Schafe ☐ Geflügel ☐ Wildtiere ☐ Kleinsäuger
    ☐ Pferde ☐ Rinder ☐ Schweine ☐ andere (bitte angeben): .....

11. Welche Aussage ist zutreffend, wenn Sie für die letzten 12 Monate den Kontakt zu Ihrem Tier beschreiben?

Ihr Tier...	Täglich	Mehrmals in der Woche	Mehrmals im Monat	Selten	Nie
a) ... darf auf dem Sofa liegen	☐	☐	☐	☐	☐
b) ... darf mit in Ihr Bett	☐	☐	☐	☐	☐
c) ... wird in Ihrer Badewanne/Dusche gewaschen	☐	☐	☐	☐	☐
d) ... wird von Ihnen gestreichelt/gekrault	☐	☐	☐	☐	☐
e) ... darf aus der Hand fressen	☐	☐	☐	☐	☐
f) ... darf Ihr Gesicht lecken	☐	☐	☐	☐	☐
g) Sonstiger Kontakt zu dem Tier (bitte angeben):	☐	☐	☐	☐	

X

... weitere Angaben zum getesteten Tier...

12. Hat sich Ihr Tier innerhalb der letzten 12 Monate in einer der folgenden Einrichtungen aufgehalten?

a) Tierpension	☐ Ja: Wann zuletzt (Monat/Jahr)? ____/____	☐ Nein
b) Tierheim	☐ Ja: Wann zuletzt (Monat/Jahr)? ____/____	☐ Nein
c) Kindertagesstätte, Schule	☐ Ja: Wann zuletzt (Monat/Jahr)? ____/____	☐ Nein
d) Tierschau/Ausstellung	☐ Ja: Wann zuletzt (Monat/Jahr)? ____/____	☐ Nein
e) Pflege-Einrichtung / Reha-Einrichtung	☐ Ja: Wann zuletzt (Monat/Jahr)? ____/____	☐ Nein
f) als Therapie-Tier eingesetzt (z.B. im Krankenhaus)	☐ Ja: Wann zuletzt (Monat/Jahr)? ____/____	☐ Nein
g) Hundeschule / Hundesport	☐ Ja: Wann zuletzt (Monat/Jahr)? ____/____	☐ Nein

13. Welches der folgenden Futtermittel frisst Ihr Tier? (Mehrfachantworten möglich)
- ☐ Nassfutter aus Dose oder Beutel
- ☐ Trockenfutter
- ☐ Trockenfleischprodukte (z.B. Schweineohr)
- ☐ Frischfleisch, roh (z.B. Pansen etc.)
- ☐ Speisereste/Tischabfälle
- ☐ Leckerlies (z.B. Kauknochen, Katzensticks etc.)
- ☐ Ergänzungsfuttermittel (z.B. Vitamin- oder Mineralstoffpräparate)
- ☐ sonstiges (bitte angeben): .....

14. Neigt Ihr Tier zu...?

a) Fressen von Kot (Koprophagie)	☐ Ja	☐ Nein	☐ unbekannt
b) Gefräßigkeit, z.B. Aufnahme von Müll (Polyphagie)	☐ Ja	☐ Nein	☐ unbekannt
c) Appetitlosigkeit (Inappetenz)	☐ Ja	☐ Nein	☐ unbekannt

15. Leidet Ihr Tier an einer akuten Erkrankung? (kurzer Krankheitsverlauf von 3-14 Tagen)
☐ Ja, und zwar: ..... ☐ Nein ☐ unbekannt

16. Leidet Ihr Tier an chronischen Erkrankungen?
(langer Krankheitsverlauf von über 14 Tagen, z.B. Diabetes, Hauterkrankung, Tumor, Epilepsie...)
☐ Ja, und zwar: ..... ☐ Nein ☐ unbekannt

17. Muss Ihr Tier regelmäßig entzündungshemmende Medikamente (z.B. Metacam®, Rimadyl®) einnehmen?
☐ Ja ☐ Nein ☐ unbekannt ☐ keine Angabe
↳ *falls ja,* seit wann? (Monat/Jahr)? ____/____

18. Muss Ihr Tier regelmäßig Magensäure-blockende Medikamente einnehmen?
(sog. Protonenpumpenhemmer, z.B. Omeprazol)
☐ Ja ☐ Nein ☐ unbekannt ☐ keine Angabe
↳ *falls ja,* seit wann? (Monat/Jahr)? ____/____

19. Wurde Ihr Tier innerhalb der letzten 3 Monate mit Antibiotika behandelt? (außer Wurmkur)
☐ Ja ☐ Nein ☐ unbekannt ☐ Keine Angabe
↳ *falls ja:* Warum? Diagnose/Erkrankung: .....
Wann? (Monat/Jahr)? ____/____
Wie? ☐ Lokale Behandlung (Salben, Augentropfen)
☐ Systemische Behandlung (Tabletten, Spritzen, o.ä.)

20. Hatte Ihr Tier in den letzten 4 Wochen Durchfall?
(Unter Durchfall verstehen wir mehr als 3 ungeformte Stühle/Tag.)
☐ Ja ☐ Nein ☐ unbekannt ☐ Keine Angabe
↳ *falls ja,* wie lange dauerte der Durchfall an?
☐ wenige Tage bis max. 3 Wochen ☐ länger als 3 Wochen ☐ keine Angabe

21. Hatte Ihr Tier innerhalb der letzten 12 Monate Kontakt zu einem Menschen oder Tier mit Durchfall?
☐ Ja ☐ Nein ☐ unbekannt ☐ Keine Angabe
↳ *falls ja,* ☐ zu einem Menschen. Wann letztmalig? (Monat/Jahr)? ____/____
☐ zu einem Tier. Wann letztmalig? (Monat/Jahr)? ____/____

22. Hatte Ihr Tier innerhalb der letzten 12 Monate einen stationären Aufenthalt in einer Tierklinik?
☐ Ja: Wann war der letzte Aufenthalt? (Monat/Jahr)? ____/____ ☐ Nein ☐ unbekannt

23. Hatte Ihr Tier innerhalb der letzten 12 Monate Kontakt zu einem anderen Patienten (Mensch oder Tier) nachdem dieser kurz zuvor in einer Klinik behandelt wurde?
☐ Ja ☐ Nein ☐ unbekannt ☐ Keine Angabe
↳ *falls ja,* ☐ zu einem Menschen. Wann letztmalig? (Monat/Jahr)? ____/____
☐ zu einem Tier. Wann letztmalig? (Monat/Jahr)? ____/____

Aufkleber Person ID

III. Angaben zum Tierhalter / zur Tierhalterin

24. In welchem Bundesland wohnen Sie? .....

25. Wie würden Sie Ihr Wohnumfeld beschreiben?
- ☐ Großstadt
- ☐ Kleinstadt
- ☐ Auf dem Land
- ☐ Keine Angabe

26. Geburtsdatum (Monat/Jahr)? ...../.....

27. Geschlecht
☐ weiblich ☐ männlich

28. In welchem Beruf bzw. Tätigkeitsfeld sind Sie aktuell tätig (Mehrfachnennungen möglich)?
- ☐ Landwirtschaft, und zwar als: .....
- ☐ Nahrungsmittelproduktion, und zwar als: .....
- ☐ Gesundheitswesen, und zwar als: .....
- ☐ anderer Tätigkeitsbereich, und zwar als: .....
- ☐ aktuell keine berufliche Tätigkeit (Elternzeit, Rente, etc.)
- ☐ keine Angabe

29. Leben in Ihrem Haushalt Kinder bis 16 Jahre?
☐ Ja ☐ Nein ☐ keine Angabe

↳ *falls ja,* bitte geben Sie an wie viele Kinder in welcher Altersgruppe sind:
Anzahl der Kinder unter 2 Jahre: ..... Kind/er
Anzahl der Kinder 2 bis 9 Jahre: ..... Kind/er
Anzahl der Kinder 10 bis 16 Jahre: ..... Kind/er

30. Lebt in Ihrem Haushalt eine Person, die an einer chronischen Erkrankung leidet?
☐ Ja ☐ Nein ☐ unbekannt ☐ keine Angabe

↳ *falls ja,* welche Erkrankung? .....

31. Lebt in Ihrem Haushalt eine Person oder ein Tier, bei der *Clostridium difficile* nachgewiesen wurde?
☐ Ja ☐ Nein ☐ unbekannt ☐ keine Angabe

↳ *falls ja,* ☐ bei einem Menschen. Wann? (Monat/Jahr)? ...../.....
☐ bei einem Tier. Wann? (Monat/Jahr)? ...../.....

32. Welche der folgenden Lebensmittel nehmen Sie zu sich?

	Täglich	Mehrmals in der Woche	Mehrmals im Monat	Selten	Nie
... Leitungswasser als Kaltgetränk	☐	☐	☐	☐	☐
... Rohmilch/-produkte	☐	☐	☐	☐	☐
... Rohfleisch/-produkte (z.B. Hackfleisch)	☐	☐	☐	☐	☐
... abgepackte Salate	☐	☐	☐	☐	☐
... probiotische Drinks (z.B. Actimel®)	☐	☐	☐	☐	☐

33. Hatten Sie innerhalb der letzten 12 Monate Kontakt zu einem Menschen oder Tier mit Durchfall?
☐ Ja ☐ Nein ☐ unbekannt ☐ keine Angabe

↳ *falls ja,* ☐ zu einem Menschen. Wann letztmalig? (Monat/Jahr)? ...../.....
☐ zu einem Tier. Wann letztmalig? (Monat/Jahr)? ...../.....

34. Hatten Sie innerhalb der letzten 12 Monate einen Klinikaufenthalt von mindestens einer Woche?
☐ Ja: Wann war der letzte Aufenthalt? (Monat/Jahr)? ...../..... ☐ Nein ☐ unbekannt ☐ keine Angabe

35. Hatten Sie Kontakt zu einem anderen Patienten (Mensch oder Tier), nachdem dieser in den letzten 12 Monaten in einer Klinik behandelt wurde?
☐ Ja ☐ Nein ☐ unbekannt ☐ keine Angabe

↳ *falls ja,* ☐ zu einem Menschen. Wann? (Monat/Jahr)? ...../.....
☐ zu einem Tier. Wann? (Monat/Jahr)? ...../.....

... weitere Angaben zum Tierhalter / zur Tierhalterin

36. Hatten Sie in den letzten 4 Wochen Durchfall?
(Unter Durchfall verstehen wir mehr als 3 ungeformte Stühle pro Tag.)
☐ Ja ☐ Nein ☐ unbekannt ☐ keine Angabe
↳ *falls ja,* wie lange dauerte der Durchfall an?
☐ wenige Tage bis max. 3 Wochen ☐ länger als 3 Wochen ☐ unbekannt

37. Nehmen Sie regelmäßig entzündungshemmende Medikamente (z.B. Aspirin®, Ibuprofen) ein?
☐ Ja ☐ Nein ☐ unbekannt ☐ keine Angabe
↳ *falls ja,* seit wann? (Monat/Jahr)? ...../.....

38. Nehmen Sie regelmäßig Magensäure-blockende Medikamente ein?
(sog. Protonenpumpenhemmer, z.B. Nexium®, Pantozol®)
☐ Ja ☐ Nein ☐ unbekannt ☐ keine Angabe
↳ *falls ja,* seit wann? (Monat/Jahr)? ...../.....

39. Hatten Sie innerhalb der letzten 12 Monate Kontakt zu einem Durchfall-Patienten (Mensch oder Tier)?
☐ Ja ☐ Nein ☐ unbekannt ☐ keine Angabe
↳ *falls ja,* ☐ zu einem Menschen. Wann letztmalig? (Monat/Jahr)? ...../.....
☐ zu einem Tier. Wann letztmalig? (Monat/Jahr)? ...../.....

40. Wurden Sie innerhalb der letzten 2 Monate mit Antibiotika behandelt?
☐ Ja ☐ Nein ☐ unbekannt ☐ Keine Angabe
↳ *falls ja:* Warum? Diagnose/Erkrankung: .....
Wann? (Monat/Jahr)? ...../.....
Wie? ☐ Lokale Behandlung (Salben, Augentropfen)
☐ Systemische Behandlung (Tabletten, Spritzen, o.ä.)

41. Wurden Sie innerhalb des letzten Jahres mit einer Chemotherapie behandelt?
☐ Ja ☐ Nein ☐ keine Angabe

42. Leiden Sie an einer chronischen Erkrankung (z.B. Diabetes, Neurodermitis, u.a.)?
☐ Ja ☐ Nein ☐ keine Angabe
↳ *falls ja,* welche Erkrankung? .....

43. Ist bei Ihnen schon einmal *Clostridium difficile* nachgewiesen worden?
☐ Ja ☐ Nein ☐ unbekannt ☐ Keine Angabe
↳ *falls ja,* hatten Sie dabei assoziierte Krankheitssymptome?
☐ Ja, und zwar: ..... ☐ Nein ☐ unbekannt ☐ keine Angabe

IV. Sonstiges

44. Dürfen wir Sie bei weiteren Fragen im Rahmen dieser Studie nochmals kontaktieren?
☐ Ja ☐ Nein

Vielen Dank für Ihre Mitarbeit!

11. Publication List

Hille, K., Möbius, N., Akmatov, M. K., Verspohl, J., Rabold, D., Hartmann, M., Günther, K., Obi, N. & Kreienbrock, L. (2014). Zoonoses research in the German National Cohort. *Bundesgesundheitsbl* **57**, 1277-1282.

Schneeberg, A., Ehricht, R., Slickers, P., Baier, V., Neubauer, H., Zimmermann, S., Rabold, D., Lubke-Becker, A. & Seyboldt, C. (2015). DNA Microarray-Based PCR Ribotyping of *Clostridium difficile*. *J Clin Microbiol* **53**, 433-442.

Rabold D., Espelage W., Abu Sin M., Eckmanns T., Schneeberg A., Neubauer H., Möbius, N., Hille, K., Wieler, L.H., Seyboldt, C., & Lübke-Becker, A. (2018). The zoonotic potential of *Clostridium difficile* from small companion animals and their owners. *PLOS ONE*. 13(2):e0193411. doi: 10.1371/journal.pone.0193411.

Presentations/Talks:

D. Rabold, W. Espelage, F. Grzebin, M. Abu-Sin, T. Eckmanns, A. Schneeberg, H. Neubauer, L. H. Wieler, A. Luebke-Becker, C. Seyboldt (2015). Human pathogenic *Clostridium difficile* strains detected in companion animals in a Germany-wide survey. *Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM) e.V., Münster – 28/09/2015; 67. Jahrestagung der DGHM e.V., 27.-30.09.2015*

D. Rabold, W. Espelage, F. Grzebin, M. Abu-Sin, T. Eckmanns, H. Neubauer, A. Schneeberg, L. H. Wieler, A. Lübke-Becker, C. Seyboldt (2016). *Clostridium difficile* in small companion animals and their owners: human pathogenic strains isolated in dogs and cats in a Germany-wide survey. *Tagung der DVG Fachgruppe Bakteriologie und Mykologie, „Mikrobiologie in der Tiermedizin GESTERN – HEUTE - MORGEN", Jena – 02/09/2016; 31.08.-02.09.2016*

C. Seyboldt, D. Rabold, A. Lübke-Becker, A. Schneeberg, T. Eckmanns, L.H. Wieler, H. Neubauer (2018). Überblick zu *Clostridium difficile* bei Hunden und Katzen. *VPH-Seminar 2018: Von Mann und Maus – Haus- und Heimtiere im One Health Kontext, 02. Februar 2018, Hannover.*

A. Lübke-Becker, D. Rabold, C. Seyboldt, B. Walther (2018). Antibiotika-resistente und zoonotische Bakterien sind Teil des Mikrobioms bei Mensch und Tier. *Symposium Antibiotikaresistenz in der Lebensmittelkette, Bundesinstitut für Risikobewertung, 08.-09.11.2018,* Berlin.

Posters:

D. Hildebrand, J. Hermes, T. Eckmanns, A. Schneeberg, H. Neubauer, L. H. Wieler, A. Lübke-Becker, C. Seyboldt (2012). Prevalence and molecular characterisation of *Clostridium difficile* in companion animals and their owners. *National Symposium on Zoonoses Research, October 11-12, 2012,* Berlin.

D. Rabold, W. Espelage, F. Grzebin, T. Eckmanns, A. Schneeberg, H. Neubauer, L. H. Wieler, A. Lübke-Becker, C. Seyboldt (2013). Prevalence, typing and molecular characterization of *Clostridium difficile* in companion animals and their owners. *National Symposium on Zoonoses Research, September 19-20, 2013,* Berlin.

D. Rabold, W. Espelage, F. Grzebin, M. Abu Sin, T. Eckmanns, N. Pfaffendorf-Regler, A. Schneeberg, H. Neubauer, L. H. Wieler, A. Lübke-Becker, C. Seyboldt (2014). Prevalence of *Clostridium difficile* and diversity of genotypes colonizing companion animals and their owners: A Germany-wide survey. *National Symposium on Zoonoses Research, October 15-16, 2014,* Berlin.

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13. Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 17.09.2019 Denise Rabold