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Clostridium difficile in Dutch animals: their presence, characteristics and similarities with human isolates

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Abstract

The presence and characteristics of *Clostridium difficile* were investigated in 839 faecal samples from seven different animal species in the Netherlands. The number of positive samples ranged from 3.4% (cattle) to 25.0% (dogs). Twenty-two different PCR ribotypes were identified. Among 96 isolates, 53% harboured toxin genes. All *C. difficile* isolates from pigs, cattle and poultry were toxinogenic, whereas the majority of isolates from pet animals consisted of non-toxinogenic PCR ribotypes 010 and 039. Ribotype 012 was most prevalent in cattle and ribotype 078 in pigs. No predominant ribotypes were present in horse and poultry samples. Overall, PCR ribotypes 012, 014 and 078 were the most frequently recovered toxinogenic ribotypes from animal samples. Comparison with human isolates from the Dutch Reference Laboratory for *C. difficile* at Leiden University Medical Centre (LUMC) showed that these types were also recovered from human hospitalized patients in 2009/2010, encompassing 0.8%, 11.4% and 9.8% of all isolates, respectively. Application of multiple-locus variable-number tandem-repeat analysis indicated a genotypic relation of animal and human ribotype 078 strains, but a clear genotypic distinction for ribotypes 012 and 014. We conclude that toxinogenic *C. difficile* PCR ribotypes found in animals correspond to PCR ribotypes associated with human disease in hospitalized patients in the Netherlands. Contrary to PCR ribotype 078, significant genetic differences were observed between animal and human PCR ribotype 012 and 014 isolates.

Keywords: Animals, *Clostridium difficile*, epidemiology, MLVA, PCR ribotyping
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Introduction

Clostridium difficile is the most important cause of hospital-associated diarrhoea, with the highest incidence rate seen in patients >65 years of age, resulting in excess mortality rates [1]. It is typically associated with antimicrobial therapy, which disrupts the colonic microbiota and stimulates growth and toxin production. Two large toxins, encoded by two separate genes, named *tcdA* (TcdA or toxin A) and *tcdB* (TcdB or toxin B) are considered to be the primary virulence factors. Additionally, some strains also produce binary toxin (CDT), consisting of two distinct protein chains, CDTa and CDTb.

Clostridium difficile has been recognized as an important emerging pathogen in both humans and animals. Characteristically, *Clostridium difficile* infection (CDI) has been considered nosocomial but a remarkable rise in the rate of community-associated (CA)-CDI has occurred [2,3], the source of which is not clearly defined. The similarity of various PCR ribotypes recovered from humans and domestic animals suggests a possible animal reservoir for human CDI [4,5]. Epidemiological research on this potential relationship, however, is limited.

Multiple-locus variable-number tandem-repeat analysis (MLVA) is regarded as a suitable method to study molecular epidemiology of *C. difficile*. Its application on PCR ribotype 078 isolates from pigs and humans revealed a high similarity [6], but other PCR ribotypes have not been investigated. The aim of this study is to determine the presence and diversity of *C. difficile* in Dutch animals and to compare the isolates for genetic relatedness to those from patients hospitalized in the Netherlands by means of MLVA.

Materials and Methods

Faecal samples were collected from healthy poultry, pigs, veal calves and dairy cattle (100 samples each) at abattoirs during 2009 and 2010 by the Dutch Food and Consumer Product Safety Authority (monitoring samples). One faecal specimen per epidemiological unit (herd or flock) was obtained from arbitrarily selected, apparently healthy animals representing the Dutch animal populations. Samples were stored in buffered peptone water with 10% glycerol (w/v) at −80°C.

Additionally, faecal specimens submitted for routine microbiological diagnostic procedures (virology, bacteriology and/or parasitology) from diarrheic animals were tested (diagnostic samples). These were collected arbitrarily during 2009 and 2010 and stored without preservatives at −20°C. Samples from dogs (n = 116), cats (n = 115), horses (n = 135), poultry (n = 21), sheep (n = 11) and dairy cattle (n = 5) were obtained from the Veterinary Microbiological Diagnostic Centre of the Faculty of Veterinary Medicine in Utrecht (VMDC); pig samples (n = 36) were collected from the Animal Health Service in Deventer (AHS).

Human isolates were collected at the National Reference Laboratory for *C. difficile* at Leiden University Medical Centre (LUMC) from patients with diarrhoea who tested positive for *C. difficile* toxin or from a surveillance study for CDI in hospitalized patients in 19 hospitals. The prevalence of human PCR ribotypes was based on 1552 samples collected from January 2009 to August 2010. MLVA data involved randomly selected PCR ribotype 012, ribotype 014 and ribotype 078 isolates from January 2006 to August 2010.

The culture method involved heat shock treatment (60′ in water at 60°C), after which samples were inoculated onto selective media, *Clostridium difficile* Selective Medium (Oxoid PB5054A; Oxoid, Basingstoke, UK) and Brazier's *Clostridium difficile* selective agar (Oxoid PB5191A). Plates were incubated anaerobically in jars (Mart; Anoxomat, Lichtenvoorde, the Netherlands) for 7 days at 37°C. Enrichment of heat shock-treated samples was performed during 7 days (1 g in 9 mL BHI broth supplemented with cycloserine-cefoxitin (*Clostridium difficile* Selective Supplement SR0096E; Oxoid, Basingstoke, UK) and 0.1% sodium taurocholate), followed by subculturing on selective agar media. At regular intervals, plates were examined for suspect colonies (morphology, typical odour and positive latex slide agglutination test (Oxoid)), which were pure cultured on Heart Infusion Sheep blood agar (HIS) and stored in buffered peptone water with 20% glycerol (w/v) at −80°C.

After identification by PCR based on the presence of the gluD gene [7], isolate characterization was based on the presence of toxin genes (*tcdA, tcdB, cdtA*, and *cdtB*) [8,9], PCR ribotyping [10] and MLVA on seven loci or, because locus A6*Cd* is absent in type 078 strains, six for ribotype 078 [6,11].

The genetic relationships among the isolates were determined by the number of differing loci and the summed absolute distance as coefficients for calculating the minimum spanning tree (MST) [12], using Bionumerics software (Version 6.01; Applied Maths NV, Sint-Martens-Latem, Belgium). Isolates with a sum tandem repeat difference (STRD) ≤10 were defined as genetically related, irrespective of the number of differing loci. Clonal complexes were defined by an STRD ≤2, provided that isolates were single locus variants (SLVs) or double locus variants (DLVs) [13].

Results

In this study, the overall isolation rate of *C. difficile* in animal samples was 11.4%. The number of positive samples varied among different animal host species, ranging from 3.4% to 25.0% (Table 1).

Among 22 identified PCR ribotypes, 16 were toxinogenic, represented by 51 isolates (53.1%) (Table 2). Non-toxino-

TABLE 1. Prevalence of *Clostridium difficile* in samples from various animal species

Animal species	Sample collection	No. samples	No. of positive samples (%)
Dogs	Diagnostics	116	29 (25.0)
Cats	Diagnostics	115	18 (15.7)
Horses	Diagnostics	135	24 (17.8)
Pigs		136	9 (6.6)
	Monitoring	100	0
	Diagnostics	36	9 (25.0)
Cattle		205	7 (3.4)
Dairy cows	Monitoring	100	1 (1.0)
	Diagnostics	5	0
Veal calves	Monitoring	100	6 (6.0)
Sheep	Diagnostics	11	2 (18.2)
Poultry		121	7 (5.8)
	Monitoring	100	5 (5.0)
	Diagnostics	21	2 (9.5)
Total samples		839	96 (11.4)
Subtotal	Monitoring	400	12 (3.0)
Subtotal	Diagnostics	439	84 (19.1)

TABLE 2. ***Clostridium difficile*** **PCR ribotypes isolated from different animal species and humans in 2009/2010 in the Netherlands**

Ribotype	Animal host species								No. of animal isolates (%)	No. of human isolates (%)
	Dog	Cat	Horse	Pig	Dairy cow	Calf	Sheep	Poultry		
Toxinogenic isolates										
001									–	381 (24.5)
002									–	81 (5.2)
003								1	1 (1.0)	9 (0.6)
005			2	1					3 (3.1)	49 (3.2)
006			1						1 (1.0)	6 (0.4)
012	2		2		1	5			10 (10.4)	13 (0.8)
014	7	1	3					2	13 (13.5)	177 (11.4)
015							1		1 (1.0)	47 (3.0)
021	1								1 (1.0)	1 (0.1)
023*			1	1					2 (2.1)	30 (1.9)
027*									–	68 (4.4)
033*						1			1 (1.0)	1 (0.1)
042			1						1 (1.0)	2 (0.1)
045*			1						1 (1.0)	20 (1.3)
056								1	1 (1.0)	25 (1.6)
078*			2	7					9 (9.4)	152 (9.8)
097							1		1 (1.0)	4 (0.3)
107	1								1 (1.0)	2 (0.1)
126*			1						1 (1.0)	33 (2.1)
Subtotal	11	1	14	9	1	6	2	4	48 (50.0)	1101 (70.9)
Non-toxinogenic isolates										
009	1	1							2 (2.1)	–
010	12	9	3					2	26 (27.1)	–
031	1								1 (1.0)	–
035			1						1 (1.0)	–
039	3	5	1						9 (9.4)	–
051			1						1 (1.0)	–
Subtotal	17	15	6	–	–	–	–	2	40 (41.7)	–
Unidentified	1	2	4					1	8 (8.3)	97 (6.3)
Other									–	354 (22.8)
Total isolates	29	18	24	9	1	6	2	7	96 (100)	1552 (100)

All isolates belonging to PCR ribotypes described as toxinogenic were positive for the presence of toxin genes *tcdA* and *tcdB* (A+B+), except for one isolate of ribotype 033 (A+B–). PCR ribotypes marked with an asterix (*) contained binary toxin genes (*CdtA*/*CdtB*).

genic ribotypes predominated in cat and dog samples (94.4% and 62.1%, respectively). Toxin genes were identified in all porcine and bovine isolates, in the majority of horse isolates (71%), and in 57% of poultry isolates. Binary toxin genes were detected in isolates from pigs (89%), horses (21%) and one calf. All isolates containing binary toxin genes also harboured toxin A and toxin B genes, except one isolate from a calf (PCR ribotype 033), which tested positive for toxin A, but negative for toxin B.

Ribotype 010 was the most common type overall (27.1% of all isolates), followed by ribotypes 014 (13.5%), 012 (10.4%), 039 (9.4%) and 078 (9.4%). These ribotypes accounted for 69.8% of all isolates. The ribotype profiles of eight (8.3%) of the animal isolates are referred to as 'unidentified' (i.e. did not match with any isolates in the established database).

The variety in ribotypes differed per host species, being highest in horses and poultry, with 13 established ribotypes and four as yet undesignated ribotypes among 24 isolates and five ribotypes among seven isolates, respectively. In contrast, six out of seven (85.7%) cattle isolates were identified as ribotype 012, while in pig samples ribotype 078 was most prevalent (seven isolates, 78%). The most frequently found human ribotypes were 001 (24.5%), 014 (11.4%), 078 (9.8%), 002 (5.2%) and 027 (4.4%).

Seven ribotypes were represented by two or more animal isolates, which were characterized by MLVA. Fig. 1 shows a minimal spanning tree (MST) of 70 *C. difficile* isolates from six animal species. Six genetically related and seven clonal complexes (CC) were identified among four PCR ribotypes (010, 012, 014 and 078). Each complex was represented by isolates of a single ribotype.

Four out of seven clonal complexes consisted of isolates from a single host species; one encompassed canine and bovine ribotype 012 isolates (CC-1), whereas two contained type 010 isolates from either dogs and poultry (CC-2) or horse and cat (CC-4). Among the six genetically related complexes, three comprised isolates from various animal species.

Of nine ribotype 078 isolates, two belonged to a single clonal complex (CC-3) of equine isolates, six belonged to a single genetically related complex of porcine isolates and one other porcine isolate was not genetically linked.

Fig. 2(a,b) demonstrates an MST based on MLVA patterns of ribotype 012 and 014 isolates, recovered from both animals and humans. In Fig. 2(a), two clonal and three

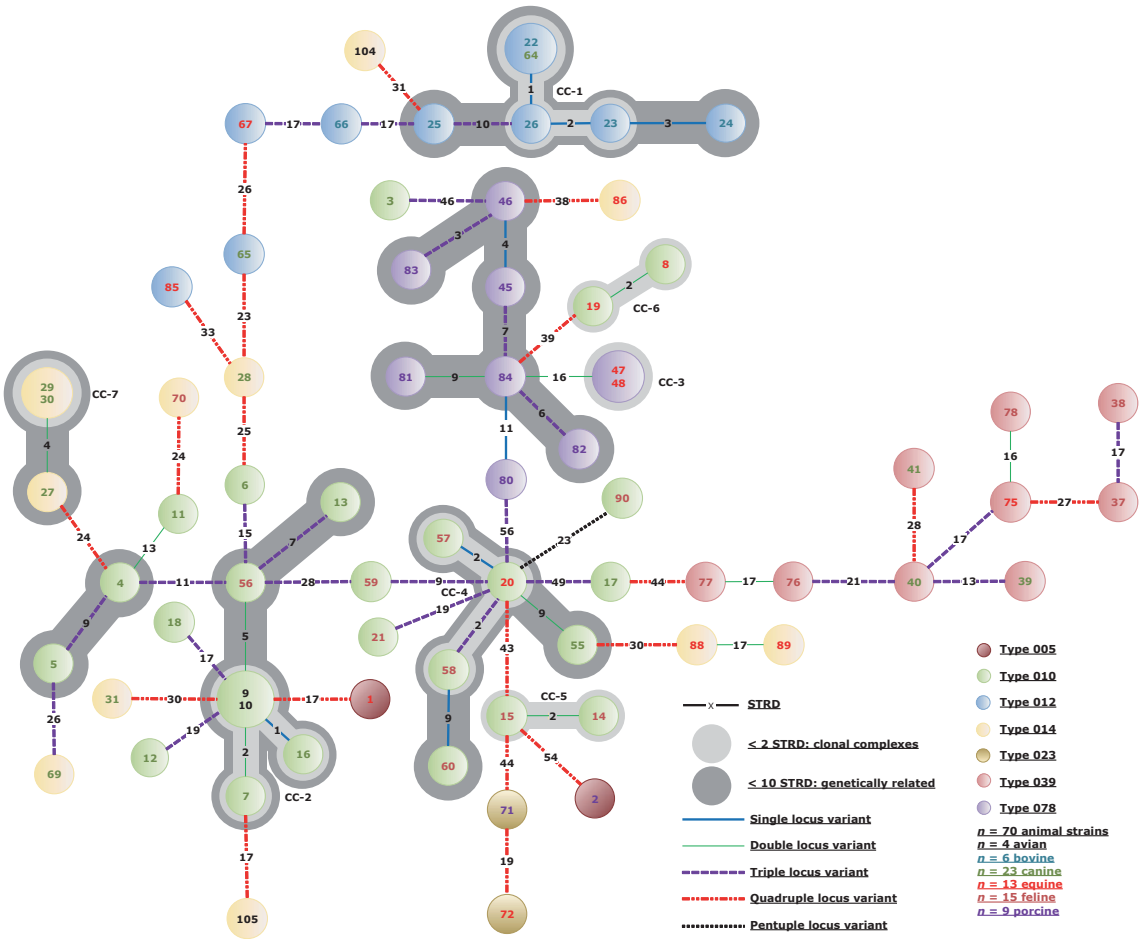

FIG. 1. Minimum spanning tree analysis of 70 *Clostridium difficile* isolates from different animal species typed by multiple-locus variable-number tandem-repeat analysis (MLVA). The numbers within the circles represent isolate identification numbers. A total of seven loci have been tested, and each circle represents either a unique isolate or isolates that are 100% homologous. The number of differences between the loci is represented by the make-up of the lines connecting the circles (fat blue line = single locus variant, thin green line = double locus variant, purple dotted line = triple locus variant, dotted red line = quadruple locus variant, and dotted black line = pentuple locus variant). The sum tandem repeat difference (STRD) between distinct isolates is displayed on the lines. Isolates with an STRD ≤2 are defined as belonging to the same clonal complex (CC) and are enveloped in light grey shade. Isolates are regarded as genetically related when showing an STRD of ≤10 (enveloped in dark grey). Each animal species is reflected in the colour of the isolate number (avian = black, bovine = light blue, canine = green, equine = red, feline = pink, and porcine = purple), while the colour of the circles depicts the PCR ribotype (type 005 = brown, type 010 = green, type 012 = blue, type 014 = yellow, type 023 = gold, type 039 = pink, and type 078 = purple).

genetically related complexes of either human or animal ribotype 012 isolates are outlined, ribotype 012 being the single most prevalent ribotype among bovine isolates (86%), and also recovered from dogs (6.9%) and horses (8.3%). Isolates belonging to PCR ribotype 014, which was the most prevalent type found in dogs (24.1%), poultry (28.6%) and horses (12.5%), appear to be more heterogeneous based on MLVA compared with PCR ribotype 012. Fig. 2(b) presents one clonal complex and two genetically related complexes, consisting either of species-specific animal isolates or human isolates.

Discussion and Conclusions

The aim of this study was to explore the presence and diversity of *C. difficile* in various animal species in the Netherlands. The wide diversity in PCR ribotypes found among horses and poultry as opposed to a limited number of ribotypes among dogs, cats, pigs and cattle is comparable to previously reported results from various countries [4,6,14–16].

In cat and dog samples, non-toxinogenic ribotype 010 was the main *C. difficile* type. The percentage of non-toxinogenic

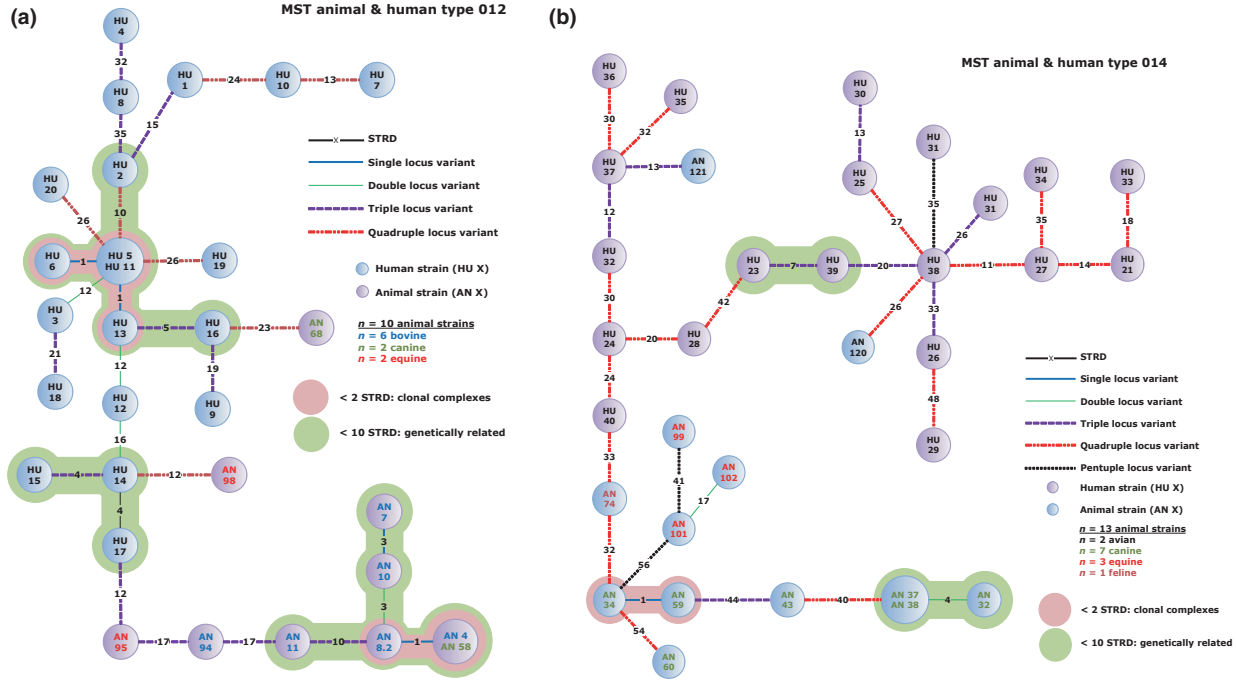

FIG. 2. Minimum spanning tree (MST) analysis of *Clostridium difficile* isolates typed by multiple-locus variable-number tandem-repeat analysis (MLVA) and recovered from human cases of CDI and from different animal species. A total of seven loci have been tested. Each circle represents either a unique isolate or isolates that are 100% homologous. The numbers within the circles represent isolate identification numbers. The number of differences between the loci is represented by the make-up of the lines connecting the circles (fat blue line = single locus variant, thin green line = double locus variant, purple dotted line = triple locus variant, dotted red line = quadruple locus variant, and dotted black line = pentuple locus variant). The sum tandem repeat difference (STRD) between distinct isolates is displayed on the lines. Isolates with an STRD ≤2 are defined as belonging to the same clonal complex (CC) and are enveloped in pink shade. Isolates are regarded as genetically related when showing an STRD of ≤10 (enveloped in green). Each animal species is reflected in the colour of the isolate number (avian = black, bovine = light blue, canine = green, equine = red, and feline = pink). (a) An MST based on PCR ribotype 012 isolates of human (n = 20) and animal origin (n = 10). (b) An MST on PCR ribotype 014 isolates of human (n = 20) and animal origin (n = 13).

isolates from dogs and cats in this study (62.1% and 94.4%, respectively) is somewhat higher than described elsewhere (up to 50%) [4,17].

As expected, in pig samples PCR ribotype 078 predominated (77.8%), being the most reported type in pigs worldwide. Among humans, the prevalence of ribotype 078 has increased since 2006 and nowadays this ribotype is one of the most common types in the Netherlands and in Europe [18]. This ribotype was also recovered from Dutch horses (8.3%). Whether these horses were housed close to pigs is unknown.

In contrast to observations from the USA and Canada [14,19], ribotype 078 isolates were not detected in cattle samples. The majority of bovine isolates consisted of ribotype 012, showing a marked genetic relatedness. Five out of six type 012 isolates, all recovered from veal calves, were part of a single genetically related complex. In the Dutch veal calf industry, calves are purchased from a wide diversity of dairy farms across Europe, and more extensive variation was expected. To confirm the strong host association found in this study, more isolates from veal calves need to be examined.

Unlike in humans, where *C. difficile* strains with truncated versions of toxin A and/or toxin B (A−B+) are regularly reported, both toxin A and toxin B (A+B+) were identified in all animal toxinogenic isolates. One exception was a PCR ribotype 033 isolate from a calf, which tested positive for toxin A and binary toxin genes, and negative for the toxin B gene. This is an interesting observation that is currently being investigated further because A+B− negative strains have not been reported previously. Avbersek *et al.* [20] recovered PCR ribotype 033 isolates with a remnant of the toxin A gene and a binary toxin gene, but these strains failed to produce either toxins A or B phenotypically and were therefore referred to as ToxA−B−.

Different sample sources were used for the collection of isolates, and this may have resulted in a bias reflecting different sampling strategies. As a consequence, the isolation

frequencies observed among different host animals may not reflect the true prevalence in the animal populations. Several factors may have contributed to the variation in isolation rates in samples from various sources, such as sample storage conditions, age of the sampled animals and prior antibiotic use. This study was not set up as a prevalence survey, and interpretation of the data must be carried out with care. However, the isolation rates in samples from food animals in the Netherlands (3.4% in cattle, 5.8% in poultry and 6.6% in pigs) are in agreement with other recent European reports, with isolation frequencies up to 3% in meat samples and 5% in samples taken from animals prior to slaughter [21,22]. Studies performed in the USA and Canada reported the presence of *C. difficile* in food animals and meat with rates up to 42% [19,23,24]. This may reflect differences in geographical and/or temporal variation in *C. difficile* prevalence, although other aspects, such as age of the sample animals, could also play a role.

Despite the limitations in sample strategy, we feel that the comparison of animal and human isolates from a restricted geographical region may help to understand the ecology of *C. difficile*. We found that the occurrence of *C. difficile* PCR ribotypes in animals is predominantly animal host specific, although shared PCR ribotypes are found among various animal species. Interestingly, almost all toxinogenic animal-related types found in this survey were also recovered from hospitalized diarrhoeal patients in the Netherlands during 2009/2010. PCR ribotypes 035 and 051 were not recovered from human samples in this particular period, although they have been found sporadically in previous years since 2005. On the other hand, ribotypes 001, 002 and 027, which are frequently detected in human patients in the Netherlands, were not detected among animals in this survey.

The corresponding presence of toxinotypes and PCR ribotypes from animal and human sources in various reports has led to the suggestion of a possible epidemiological relation between human CDI and animals [4,5,25,26], although transmission from food animals or foods to humans has never been documented [27,28].

In this study, an evident overlap was seen with regard to PCR ribotypes 078, 012 and 014. The previously shown genetic relatedness between porcine and human PCR ribotype 078 isolates [6,29] was confirmed in this study; four out of seven porcine isolates were genetically related to human 078 isolates (data not shown). In contrast, the genetic distances of >12 by MLVA between human and animal ribotype 012 and 014 isolates suggests different population dynamics for distinct ribotypes with variable zoonotic potential. Comparative genomic studies have demonstrated the complex nature of interspecies transmission, including animal to human transmission and *vice versa* [26,30].

MLVA appears to have superior discriminatory power compared with other typing techniques, but its applicability in investigating zoonotic risks should be accompanied with extensive epidemiological surveys [11,13]. Although the human and animal isolates in this study originate from a single country and were recovered in the same period of time, for confident assessment of the risk of interspecies transmission more extensive studies are needed with careful consideration of study populations and more detailed information about the geographical and temporal relationship between human and animal samples.

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Transparency Declaration

The authors declare no potential conflicts of interest.

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