Molecular characterization of pathogenic Clostridium difficile strains

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Relatedness of human and animal *Clostridium difficile* PCR Ribotype 078 isolate determined on the basis of Multilocus Variable-Number tandem repeat Analysis and tetracycline resistance

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Abstract

One hundred and two human and 56 porcine *Clostridium difficile* PCR Ribotype (RT) 078 strains from four European countries were investigated by an optimized Multiple Locus Variable number tandem repeat Analysis (MLVA) and for tetracycline susceptibility. Eighty-five percent of all isolates were genetically related, irrespective of human or porcine origin. Human strains were significantly more resistant to tetracycline than porcine strains. All tetracycline resistant strains contained the Tn*916*-like transposon, harboring the *tet*(M) gene. We conclude that strains from human and porcine origin are genetically related, irrespective of country of origin. Further studies are needed to clarify the genetic relatedness of *C. difficile* RT 078 strains, including whether this is a consequence of less natural variability in this ribotype versus other ribotypes.

Introduction

Recently, we reported that *Clostridium difficile* PCR Ribotype (RT) 078 is an increasing cause of *Clostridium difficile* Infections (CDI) in humans in the Netherlands with similar disease severity as the hypervirulent RT 027 (1). Also the incidence of CDI in England caused by RT 078 has increased (2). In addition, recent studies have demonstrated that RT 078 is the predominant type in cattle and pigs (3,4). In the Netherlands, we have noticed an overlap in the occurrence of human CDI cases caused by RT 078 and the distribution of pig farms in the eastern part of the country (1). This suggests a possible link between human and porcine RT 078 strains.

To investigate the relatedness between human and porcine RT 078 strains, we applied a Multiple locus variable number tandem repeat analysis (MLVA) developed for *C. difficile* on a collection of RT 078 isolates (1,5,6). This MLVA has been proven to be more discriminatory than other genotyping methods (7,8). Since it has been suggested that the wide dissemination of *Staphylococcus aureus* ST398 in pigs and humans is associated with the frequent usage of tetracycline in pig farms, we also investigated the susceptibility to tetracycline and the genetic origin of tetracycline resistance (9-11).

Clostridium difficile strains

A total of 102 human and 56 porcine RT 078 strains were available for this study. Table 1 depicts the location of isolation and the year of isolation of each strain. All human RT 078 strains were recovered from diarrhoeal patients. The "Leeds collection" (n=67) consisted of 44 strains originating from an outbreak in Northern Ireland, 20 strains from other parts of the UK and 3 strains originating from Ireland. The "Leiden collection" consisted of 34 strains of endemic cases in the year 2006-2007. The 56 porcine strains were collected from 11 Dutch pig farms in the years 2006, 2007 and 2009. All pig farms had persistent problems of neonatal diarrhoea. Forty-seven (84%) isolates were recovered from diarrhoeal piglets.

Modification of MLVA

MLVA was adjusted for RT 078 due to the lack of specific PCR products for loci $A6_{Cd}$, $B7_{Cd}$, $C6_{Cd}$ and $G8_{Cd}$. Sequence analysis of 7 human and 8 porcine RT 078 strains revealed multiple mismatches in the primer annealing sites for loci $B7_{Cd}$, $C6_{Cd}$ and $G8_{Cd}$ and the absence of locus $A6_{cd}$. We adjusted the magnesium chloride concentration (4 mM) and annealing temperatures for loci $B7_{Cd}$, $G8_{Cd}$ (47°C) and locus $C6_{Cd}$ (46°C). All MLVA PCRs were performed in a singleplex format. MLVA

PCRs for the other loci and the analysis of the MLVA data were performed with the previously described conditions (5). The calculated Variable Number of Tandem Repeats (VNTR) of the adjusted MLVA was in complete concordance with the manually measured VNTR. The absence of locus $A6_{Cd}$ could theoretically result in less discriminative power of the MLVA. Therefore, we reanalyzed the MLVA on previously typed RT 027 (n=57) and 017 (n=71) strains (5,6). This reanalysis based on 6 loci resulted in equal numbers of Genetically related Clusters (GCs) and Clonal Complexes (CCs). Subsequently, we concluded that the optimized MLVA for RT 078, based on 6 loci is capable to discriminate between strains from various countries and origins.

Tetracycline susceptibility

All strains were tested for their susceptibility to tetracycline. The breakpoint for tetracycline was defined as MIC ≥ 8 mg/l (12). Seventy-five of the 102 human and 15 of the 56 porcine strains were resistant to tetracycline. Tetracycline resistance was not found among randomly selected isolates of the 2 most common human types 001 (n=10) and 014 (n=10). There were significantly more human strains resistant to tetracycline compared to porcine strains ($p<0.005$ measured by Chi-squared method). This difference could be explained by the fact that we included 44 outbreak strains. Thirty-eight (86%) of the outbreaks strains were resistant to tetracycline versus 15 (65%) resistant strains originating from other parts of the UK and Ireland.

The origin of tetracycline resistance was investigated by detection of mobile elements Tn*5397*-like and Tn*916*-like transposons as previously described (13). All tetracycline resistant strains contained the Tn*916*-like transposon, harboring the *tet*(M) gene. Filtermating experiments demonstrated the transfer of the Tn*916*-like transposon from a donor strain to a recipient strain. We did not detect either of the transposons in tetracycline susceptible strains. This observation suggests a high degree of relatedness of human and porcine isolates and is in agreement with recently published findings (13). Although, we cannot exclude horizontal transfer of the Tn*916*-like transposon since this transposon is widely distributed in Gram-positive bacteria and additional data are required from tetracycline resistant non-078 RTs (14-16). Recent publications show that tetracycline resistance is predominantly present in RTs 012, 017, 046 and 078 (12,17,18). A screening of randomly selected strains of these types demonstrated that tetracycline resistance in RT 012 and 046 is encoded by the Tn*5397*-like transposon, whereas the origin of tetracycline resistance in RT 017 and 078 is encoded by the Tn*916*-like transposon.

Application of optimized MLVA

Table 1 depicts the results of the MLVA of each strain per locus. A minimal spanning tree (MST) was constructed to determine the genetic relationships among strains as previously described (Figure 1) (1). In total, 116 strains belonged to one of the GCs, defined as a Summed Tandem Repeat difference (STRD) ≤ 10. The largest GC (green cluster) contained 103 strains, encompassing 47 porcine strains, 41 Leeds collection strains and 15 Leiden collection strains. Fifty-five strains were

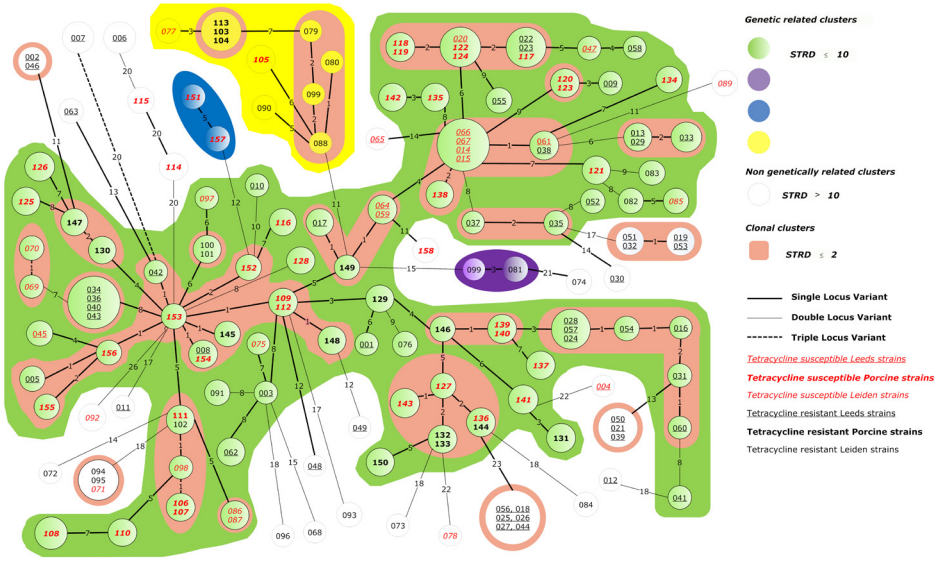

Figure 1: Minimum spanning tree analysis of 158 *C. difficile* RT 078 isolates by MLVA: Each circle represents either a unique isolate or more isolates that have identical MLVA types. The numbers between the circles represent the summed tandem repeat difference (STRD) between MLVA Types. Thick lines represent single locus variants, thin lines represent double locus variants and the interrupted lines represent triple locus variance between MLVA types. Clonal clusters are defined by a STRD ≤ 2 and genetically related clusters are defined by a STRD ≤ 10. Porcine (n=56) isolates susceptible are printed in ***red italic bold font***, human isolates susceptible to tetracycline from the Leeds collection are printed in *red italic underlined font* and human isolates from the Leiden collection susceptible to tetracycline are printed in *red italic normal font*. Isolates resistant to tetracycline from the Leeds collection are printed in black underlined font. Porcine isolates resistant to tetracycline are printed in **bold font** and human isolates from the Leiden collection are printed in normal font. The numbers represent isolates from different geographical locations: The Leeds collection: Northern Ireland 1-44, Yorkshire and Humber 45-53, South West England 54-57, North West England 58-62, East England 63-64, Ireland 65-67. The Leiden collection: Noord Holland 68-76, Zuid Holland 77-79, Utrecht 80-87, Gelderland 88-91, Brabant 92-93, Groningen 94-95, Friesland 96, Flevoland 97, Limburg 98, Overijssel 99-101 and Belgium 102. Porcine strains 103-158.

susceptible and 48 strains were resistant to tetracycline. The yellow GC contained only strains which originated in the Netherlands, encompassing both tetracycline susceptible and resistant strains. The blue GC contained only porcine strains susceptible to tetracycline which originated from one pig farm. The last GC (purple) contained only tetracycline resistant strains from the Leiden collection. Sixteen of the 23 recognized CCs (defined as a STRD ≤2) belonged to the largest GC, whereas the other 7 CCs were either single or double locus variants of the largest GC. Five CCs contained only porcine strains and 13 CCs contained only human strains, of which 8 CCs derived from the Leeds collection and 5 CCs derived from the Leiden collection. The remaining 5 CCs contained both porcine and human strains, irrespective of origin of country. Two CCs contained human strains isolated in different locations from a specific region. Nine of the 23 CCs contained outbreak strains and strains from distinct settings. Interestingly, 12 CCs contained only strains resistant to tetracycline and 8 CCs contained both tetracycline susceptible and resistant strains. The remaining 3 CCs contained only tetracycline susceptible strains. In total, 4 MLVA profiles could be recognized that contained both human and porcine strains. Overall, the MST could not differentiate between geographical origin or tetracycline phenotype, irrespective of human or porcine origin.

Table 1: Depicts of each strain the results of the optimized MLVA for each of the 7 loci ($A6_{Cd}$, $B7_{Cd}$, $C6_{Cd}$, $G8_{Cd}$ $E7_{Cd}$, $F3_{Cd}$ and $H9_{Cd}$).

MST no.	location of isolation (anonymised)	year of isolation	$A6_{Cd}$	$B7_{Cd}$	$C6_{Cd}$	$E7_{Cd}$	$F3_{Cd}$	$G8_{Cd}$	$H9_{Cd}$
001	A	2008	n/a	19	28	8	4	4	2
002	B	2008	n/a	18	26	8	4	4	2
003	E	2008	n/a	24	34	8	4	4	2
004	E	2008	n/a	13	18	6	4	4	2
005	B	2008	n/a	15	33	8	4	3	2
006	H	2008	n/a	26	27	12	4	4	2
007	GP	2008	n/a	17	49	7	4	1	2
008	I	2008	n/a	16	32	8	4	4	2
009	J	2008	n/a	22	30	8	4	6	2
010	A	2008	n/a	16	26	5	4	6	2
011	M	2008	n/a	25	25	8	4	4	2
012	T	2008	n/a	18	29	5	4	0	2
013	U	2008	n/a	21	39	5	4	6	2
014	GP	2008	n/a	21	38	8	4	3	2
015	W	2008	n/a	21	38	8	4	3	2

MST no.	location of isolation (anonymised)	year of isolation	$A6_{Cd}$	$B7_{Cd}$	$C6_{Cd}$	$E7_{Cd}$	$F3_{Cd}$	$G8_{Cd}$	$H9_{Cd}$
016	X	not supplied	n/a	19	39	5	4	4	2
017	GP	2008	n/a	21	34	8	4	5	2
018	M	2008	n/a	37	36	8	4	4	2
019	AA	not supplied	n/a	6	42	6	4	5	2
020	T	not supplied	n/a	21	32	8	4	3	2
021	E	2008	n/a	19	24	5	4	4	2
022	GP	2008	n/a	19	32	8	4	3	2
023	AD	2008	n/a	19	32	8	4	3	2
024	E	2008	n/a	19	38	5	4	5	2
025	E	2008	n/a	37	36	8	4	4	2
026	GP	2008	n/a	37	36	8	4	4	2
027	GP	2008	n/a	37	36	8	4	4	2
028	M	2008	n/a	19	38	5	4	5	2
029	AA	not supplied	n/a	21	39	5	4	6	2
030	AH	2008	n/a	35	43	5	4	5	2
031	M	2008	n/a	19	37	5	4	4	2
032	M	2008	n/a	6	43	6	4	5	2
033	GP	2008	n/a	21	41	5	4	6	2
034	AI	2008	n/a	24	33	8	4	4	2
035	AJ	2008	n/a	21	43	5	4	5	2
036	E	2008	n/a	24	33	8	4	4	2
037	AJ	2008	n/a	21	43	5	4	3	2
038	AI	2008	n/a	21	39	8	4	3	2
039	AK	2008	n/a	19	24	5	4	4	2
040	AI	2008	n/a	24	33	8	4	4	2
041	AK	2008	n/a	20	45	5	4	0	2
042	M	2008	n/a	17	33	8	4	4	2
043	M	2008	n/a	24	33	8	4	4	2
044	M	2008	n/a	37	36	8	4	4	2
045	K	2008	n/a	15	23	8	4	5	2
046	D	not supplied	n/a	18	26	8	4	4	2
047	G	2008	n/a	19	32	8	4	8	2
048	G	2008	n/a	28	34	8	4	4	2
049	C	2007	n/a	20	33	8	4	3	2
050	G	2008	n/a	19	24	5	4	4	2
051	AB	2007	n/a	6	43	6	4	5	2
052	AC	2007	n/a	15	43	7	4	5	2

MST no.	location of isolation (anonymised)	year of isolation	$A6_{Cd}$	$B7_{Cd}$	$C6_{Cd}$	$E7_{Cd}$	$F3_{Cd}$	$G8_{Cd}$	$H9_{Cd}$
053	D	2007	n/a	6	42	6	4	5	2
054	F	2008	n/a	19	39	5	4	5	2
055	Y	2007	n/a	29	32	8	4	0	2
056	Y	2007	n/a	37	36	8	4	4	2
057	AF	2008	n/a	19	38	5	4	5	2
058	L	2007	n/a	15	32	8	4	8	2
059	V	2007	n/a	21	34	8	4	3	2
060	Z	2007	n/a	20	37	5	4	4	2
061	AE	1953	n/a	21	38	8	4	3	2
062	AL	not supplied	n/a	24	26	8	4	4	2
063	O	2007	n/a	17	43	5	4	4	2
064	S	2008	n/a	21	34	8	4	3	2
065	P	not supplied	n/a	35	38	8	4	3	2
066	Q	2008	n/a	21	38	8	4	3	2
067	R	2008	n/a	21	38	8	4	3	2
068	AL	2006	n/a	26	38	12	2	4	8
069	AM	2007	n/a	26	33	10	2	4	9
070	AM	2007	n/a	26	33	11	2	4	9
071	AL	2007	n/a	29	40	9	2	4	8
072	AN	2007	n/a	24	41	9	2	4	8
073	AO	2007	n/a	25	35	13	2	4	8
074	AP	2007	n/a	20	20	12	2	4	8
075	AZ	2006	n/a	26	34	11	2	4	8
076	AZ	2006	n/a	21	34	12	2	4	9
077	AV	2007	n/a	21	37	12	2	4	8
078	BB	2007	n/a	31	35	11	2	4	8
080	AQ	2006	n/a	23	30	9	2	4	8
081	BE	2006	n/a	20	41	12	2	4	8
082	BF	2006	n/a	22	31	10	2	4	8
083	BG	2006	n/a	24	31	11	2	4	8
084	BE	2007	n/a	31	36	7	2	4	8
085	BG	2006	n/a	27	31	10	2	4	8
086	BG	2006	n/a	23	33	9	2	4	8
087	BG	2006	n/a	23	33	9	2	4	8
088	AR	2006	n/a	23	30	11	2	4	8
089	AU	2007	n/a	25	39	12	2	4	8
090	BA	2006	n/a	23	25	11	2	4	8

MST no.	location of isolation (anonymised)	year of isolation	$A6_{Cd}$	$B7_{Cd}$	$C6_{Cd}$	$E7_{Cd}$	$F3_{Cd}$	$G8_{Cd}$	$H9_{Cd}$
091	BJ	2006	n/a	26	32	10	2	4	8
092	AT	2007	n/a	18	52	11	2	4	8
093	BI	2006	n/a	27	34	12	2	4	8
094	AY	2007	n/a	29	40	9	2	4	8
095	AX	2007	n/a	29	40	9	2	4	8
096	AW	2007	n/a	26	43	13	2	4	8
097	BC	2006	n/a	19	28	10	2	4	8
098	BD	2007	n/a	18	32	9	2	4	8
099	BP	2006	n/a	23	41	12	2	4	8
100	BK	2007	n/a	18	33	10	2	4	8
101	BK	2007	n/a	18	33	10	2	4	8
102	AS	2005	n/a	18	33	9	2	4	8
103	BL	2006	n/a	21	37	9	2	4	8
104	BL	2006	n/a	21	37	9	2	4	8
105	BM	2007	n/a	23	36	11	2	4	8
106	BN	2007	n/a	19	32	9	2	4	8
107	BN	2007	n/a	19	32	9	2	4	8
108	BN	2007	n/a	13	25	9	2	4	8
109	BN	2007	n/a	18	34	4	2	4	8
110	BN	2007	n/a	13	32	9	2	4	8
111	BN	2007	n/a	18	33	9	2	4	8
112	BN	2007	n/a	18	34	4	2	4	8
113	BO	2007	n/a	21	37	9	2	4	8
114	BR	2009	n/a	8	43	8	4	4	2
115	BR	2009	n/a	28	43	8	4	4	2
116	BS	2009	n/a	25	33	8	4	6	2
117	BS	2009	n/a	21	32	8	4	3	2
118	BS	2009	n/a	25	32	8	4	3	2
119	BS	2009	n/a	25	32	8	4	3	2
121	BS	2009	n/a	23	31	8	4	3	2
122	BS	2009	n/a	23	32	8	4	3	2
123	BS	2009	n/a	24	30	8	4	3	2
124	BS	2009	n/a	23	32	8	4	3	2
125	BT	2009	n/a	20	45	8	4	4	2
126	BT	2009	n/a	20	44	8	4	4	2
127	BT	2009	n/a	16	38	8	4	4	2
128	BT	2009	n/a	18	40	8	4	5	2

MST no.	location of isolation (anonymised)	year of isolation	$A6_{Cd}$	$B7_{Cd}$	$C6_{Cd}$	$E7_{Cd}$	$F3_{Cd}$	$G8_{Cd}$	$H9_{Cd}$
129	BU	2009	n/a	21	34	8	4	4	2
130	BU	2009	n/a	18	37	8	4	4	2
131	BU	2009	n/a	15	35	8	4	4	2
132	BU	2009	n/a	16	35	8	4	4	2
133	BU	2009	n/a	16	35	8	4	4	2
134	BV	2009	n/a	16	39	8	4	3	2
135	BV	2009	n/a	15	38	8	4	3	2
136	BV	2009	n/a	16	36	8	4	4	2
137	BV	2009	n/a	28	38	8	4	5	2
138	BV	2009	n/a	21	38	8	4	3	2
139	BV	2009	n/a	21	38	8	4	5	2
140	BV	2009	n/a	21	38	8	4	5	2
141	BV	2009	n/a	15	38	8	4	4	2
142	BV	2009	n/a	15	35	8	4	3	2
143	BV	2009	n/a	16	38	8	4	5	2
144	BW	2009	n/a	16	36	8	4	4	2
145	BW	2009	n/a	17	33	8	4	4	2
146	BW	2009	n/a	21	38	8	4	4	2
147	BW	2009	n/a	20	37	8	4	4	2
148	BW	2009	n/a	17	34	8	4	4	2
149	BW	2009	n/a	23	34	8	4	4	2
150	BW	2009	n/a	11	35	8	4	4	2
151	BX	2009	n/a	8	35	8	4	1	2
152	BX	2009	n/a	18	33	8	4	6	2
153	BX	2009	n/a	18	33	8	4	4	2
154	BX	2009	n/a	18	32	8	4	4	2
155	BX	2009	n/a	20	33	8	4	3	2
156	BX	2009	n/a	18	33	8	4	3	2
157	BX	2009	n/a	8	35	8	4	6	2
158	BX	2009	n/a	12	34	8	4	3	2

All strains have a MST number which corresponds to the number used in the MST (Figure 1); geographical location: Northern Ireland 1-44, Yorkshire and Humber 45-53, South West England 54-57, North West England 58-62, East England 63-64, Ireland 65-67. Noord Holland 68-76, Zuid Holland 77-79, Utrecht 80-87, Gelderland 88-91, Brabant 92-93, Groningen 94-95, Friesland 96, Flevoland 97, Limburg 98, Overijssel 99-101 and Belgium 102. Porcine strains 103-158. For each strain an anonymised location (institute or pigfarm) of isolation, year of isolation and the MLVA results for each locus is described.

Conclusions

The suggested high relatedness between human and porcine RT 078 strains is in concordance with earlier publications based on MLVA, whole-genome analysis and Multi-Locus Sequence typing (19-22). The relatedness between human and porcine RT 078 strains in this study could be an indication of a common source as suggested in previous publications (1,19,20). However, the geographical locations of some related isolates are very distinct and can not logically be explained by any direct epidemiological link. A possible common source of RT 078 could be further supported by the observation that all tetracycline resistant strains contain the mobile element Tn*916*-like transposon which has also been described for tetracycline resistant enterococci from human and porcine origin (13). Interspecies transmission or transmission through meat is suggested as sources of infection but these are not yet established (23-25). However, data of a direct epidemiological link between human and porcine strains in this study is lacking. We also need to consider that the high relatedness between human and porcine RT 078 strains could be the consequence of less natural variability in RT 078 than in other types. A limitation of this study is the inclusion of porcine strains from only one country, while human strains derived from 4 European countries. Further studies are needed to investigate the possible transmission routes between humans and animals.

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