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Whole-genome analysis of *Clostridioides difficile* strains isolated from horses in Japan

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INTRODUCTION

Clostridioides difficile has been recognized as an important cause of acute enterocolitis in horses (1). At the 5th ICDS, we reported the observation of various PCR ribotypes (RTs) in cases of *C. difficile* infection (CDI) of thoroughbred racehorses at a racehorse clinic in Japan (8). Some of the *C. difficile* isolates from these cases had the same RT as those in humans, such as the hypervirulent RT 027 and 078. This raises the possibility of *C. difficile* as a potential zoonotic agent. We conducted whole-genome sequencing (WGS) analysis of equine *C. difficile* isolates in Japan and compared the sequences with a number of other *C. difficile* genome sequences.

MATERIALS AND METHODS

Bacterial strains

We tested 34 *C. difficile* isolates obtained from 32 horses. The isolates were obtained from feces or intestinal contents collected between May 2010 and July 2016. All isolates were toxin-producing strains as confirmed by PCR (4–5, 10).

PCR ribotyping

PCR ribotyping was performed according to the modified Stubbs method described by Kato *et al.* (6, 9).

Determination of minimum inhibitory concentration (MIC)

MICs of metronidazole, minocycline, azithromycin, and moxifloxacin were determined by a microbroth dilution method in accordance with the CLSI standard for anaerobic bacteria (2) using a commercial customized panel (Eiken Chemical). The MIC of vancomycin was determined by a concentration gradient diffusion assay (Etest, bioMérieux) in accordance with the manufacturer's instructions. Breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used for vancomycin, metronidazole, and moxifloxacin (3). No breakpoint criteria were available for minocycline and azithromycin with *C. difficile*.

Whole-genome analysis and computer-based molecular epidemiological analysis

The isolates were sequenced on an Illumina NextSeq 500 sequencer, with 612 publicly available *C. difficile* genome sequences as references. Computer-based multi-locus sequence typing (MLST) and antimicrobial-resistance gene analysis were conducted on a web-based genome analyzer (GenEpid-J, 11). The core genome single nucleotide polymorphism (SNP)-based phylogenetic tree was constructed by the approximately-maximum-likelihood phylogenetic method in FastTree (7). Quinolone-resistance-determining regions (QRDRs) *gyrA* and *gyrB* of moxifloxacin-resistant isolates were sequenced (9).

RESULTS AND DISCUSSION

Table 1. Molecular genotyping, toxins produced, antimicrobial resistance genes or mutations detected, and susceptibility to 5 antimicrobials of 34 equine isolates of *C. difficile*.

Isolate	PCR ribotype	MLST	Toxin produced	Antimicrobial resistance gene or mutation	MIC (μg/ml)				
					Metronidazole	Vancomycin	Minocycline	Azithromycin	Moxifloxacin
anaero-115	078	ST11	$A^+B^+CDT^+$	*aadE, aph(2'')-Ic, tet(M)*	≤1	1	2	4	1
anaero-125	078	ST11	$A^+B^+CDT^+$	*aadE, tet(M)*	≤1	0.5	2	4	1
anaero-145	078	ST11	$A^+B^+CDT^+$	*aadE, aph(2'')-Ic, tet(M)*	≤1	0.5	2	4	1
anaero-160	078	ST11	$A^+B^+CDT^+$	*aadE, aph(2'')-Ic, tet(M)*	≤1	1	2	4	1
anaero-162	078	ST11	$A^+B^+CDT^+$	*aadE, aph(2'')-Ic, tet(M)*	≤1	0.5	2	4	1
anaero-173	078	ST11	$A^+B^+CDT^+$	*aadE, aph(2'')-Ic, tet(M)*	≤1	1	2	4	1
anaero-179	hnc08162	ST53	$A^+B^+CDT^-$		≤1	1	≤0.5	2	1
anaero-180	078	ST11	$A^+B^+CDT^+$	*aadE, tet(M)*	≤1	1	2	4	1
anaero-198	078	ST11	$A^+B^+CDT^+$	*aadE, aph(2'')-Ic, tet(M)*	≤1	1	2	2	1
anaero-206	014	ST4	$A^+B^+CDT^-$	*tet(M)*	≤1	1	4	2	1
anaero-207	km0429	ST14	$A^+B^+CDT^-$		≤1	1	≤0.5	1	1
anaero-209	078	ST11	$A^+B^+CDT^+$	*aadE, aph(2'')-Ic, tet(M)*	≤1	1	2	4	1
anaero-212	002	ST8	$A^+B^+CDT^-$		≤1	1	≤0.5	4	1
anaero-213	014	ST2	$A^+B^+CDT^-$		≤1	1	≤0.5	1	1
anaero-224	014	ST14	$A^+B^+CDT^-$		≤1	1	≤0.5	1	1
anaero-228	027	ST1	$A^+B^+CDT^+$		≤1	1	≤0.5	1	1
anaero-229	rh13124	ST47	$A^+B^+CDT^+$		≤1	1	≤0.5	2	1
anaero-230	014	ST2	$A^+B^+CDT^-$		≤1	1	≤0.5	2	1
anaero-251	056	ST58	$A^+B^+CDT^-$		≤1	1	≤0.5	2	1
anaero-276	j41	ST36	$A^+B^+CDT^-$		≤1	0.5	≤0.5	4	2
anaero-277	014	ST3	$A^+B^+CDT^-$		≤1	0.5	≤0.5	4	1
anaero-280	c056	ST58	$A^+B^+CDT^-$		≤1	1	≤0.5	1	1
anaero-284	017	ST37	$A^-B^+CDT^-$	*aac(6')-aph(2'')*, *aadE*, *tet(M)*, *erm(B)*, *gyrA* (Thr82→Ile) *gyrB* (Ser416→Ala)	≤1	1	4	>256	>32
anaero-285	017	ST37	$A^-B^+CDT^-$	*aac(6')-aph(2'')*, *aadE*, *tet(M)*, *erm(B)*, *gyrA* (Thr82→Ile) *gyrB* (Ser416→Ala)	≤1	0.5	4	>256	>32
anaero-286	c056	ST34	$A^+B^+CDT^-$		≤1	1	≤0.5	2	1
anaero-288	002	ST8	$A^+B^+CDT^-$		≤1	0.5	≤0.5	4	1
anaero-302	078	ST11	$A^+B^+CDT^+$	*aac(6')-aph(2'')*, *tet(40)*, *tet(O)*, *gyrA* (Thr82→Ile)	≤1	0.5	≤0.5	2	>32
anaero-303	078	ST11	$A^+B^+CDT^+$	*aac(6')-aph(2'')*, *tet(40)*, *tet(O)*, *gyrA* (Thr82→Ile)	≤1	0.5	≤0.5	2	>32
anaero-313	017	ST37	$A^-B^+CDT^-$	*aac(6')-aph(2'')*, *aadE*, *tet(M)*, *erm(B)*	≤1	0.5	4	>256	2
anaero-323	017	ST37	$A^-B^+CDT^-$	*aac(6')-aph(2'')*, *aadE*, *tet(M)*, *erm(B)*, *gyrA* (Thr82→Ile)	≤1	0.5	2	>256	>32
anaero-342	078	ST11	$A^+B^+CDT^+$	*tet(M)*	≤1	1	4	8	2
anaero-343	078	ST11	$A^+B^+CDT^+$	*tet(M)*	≤1	1	4	2	2
anaero-349	017	ST37	$A^-B^+CDT^-$	*aac(6')-aph(2'')*, *aadE*, *tet(M)*, *erm(B)*	≤1	1	2	>256	2
anaero-350	078	ST11	$A^+B^+CDT^+$	*tet(M)*	≤1	1	4	2	1

Relation between PCR ribotype and MLST sequence type (Table 1)

The isolates were classified into 11 RTs and 13 MLST types. Ribotype corresponded to MLST type in all isolates except RT 014 and RT c056.

Core genome SNP-based phylogenetic analysis (Figure 1)

- The RT 078 isolates were divided into 3 sublineages which corresponded to their geographical and temporal differences.
  - ➢ One lineage was associated with a nosocomial outbreak in a clinic.
- Isolates in most RTs (especially RT 078, RT 027, and RT 017) in horses were indistinguishable from isolates from humans and bovines despite host and geographical differences among them.
  - ➢ High genetic relatedness between equine and clinical isolates indicates the possibility of transmission of *C. difficile* between horses and humans.
- RT rh13124 ($A^+B^+CDT^+$) was closely related to RT 027.

Antimicrobial susceptibility and resistance gene detected by GenEpid-J (Table 1)

- All equine isolates were susceptible to metronidazole (MIC: ≤1 μg/ml) and vancomycin (MIC: 0.5–1 μg/ml).
- *tet(M)* was detected in all RT 078 and RT 017 isolates and one RT 014 isolate. These isolates had reduced susceptibility to minocycline. In contrast, the presence of *tet(40)* and *tet(O)* did not seem to influence susceptibility. *erm(B)* was detected in all RT 017 isolates with reduced susceptibility to azithromycin.
- Two RT 078 and three RT 017 isolates were resistant to moxifloxacin. The same nucleotide mutation (T245→C) corresponding to an amino acid substitution (Thr82→Ile) was observed in QRDR *gyrA.* Another amino acid substitution (Ser416→Ala) was detected in the two RT 078 isolates.
- Aminoglycoside-resistance genes (*aac(6')-aph(2'')*, *aadE*, and *aph(2'')-Ic*) were detected in some isolates despite *C. difficile*'s natural resistance to aminoglycosides.
  - ➢ No isolates with low susceptibility to metronidazole and vancomycin were observed.
  - ➢ The presence of the resistance genes and mutations seemed to be related to more to RT than to the consumption of these agents in equine practice in Japan.

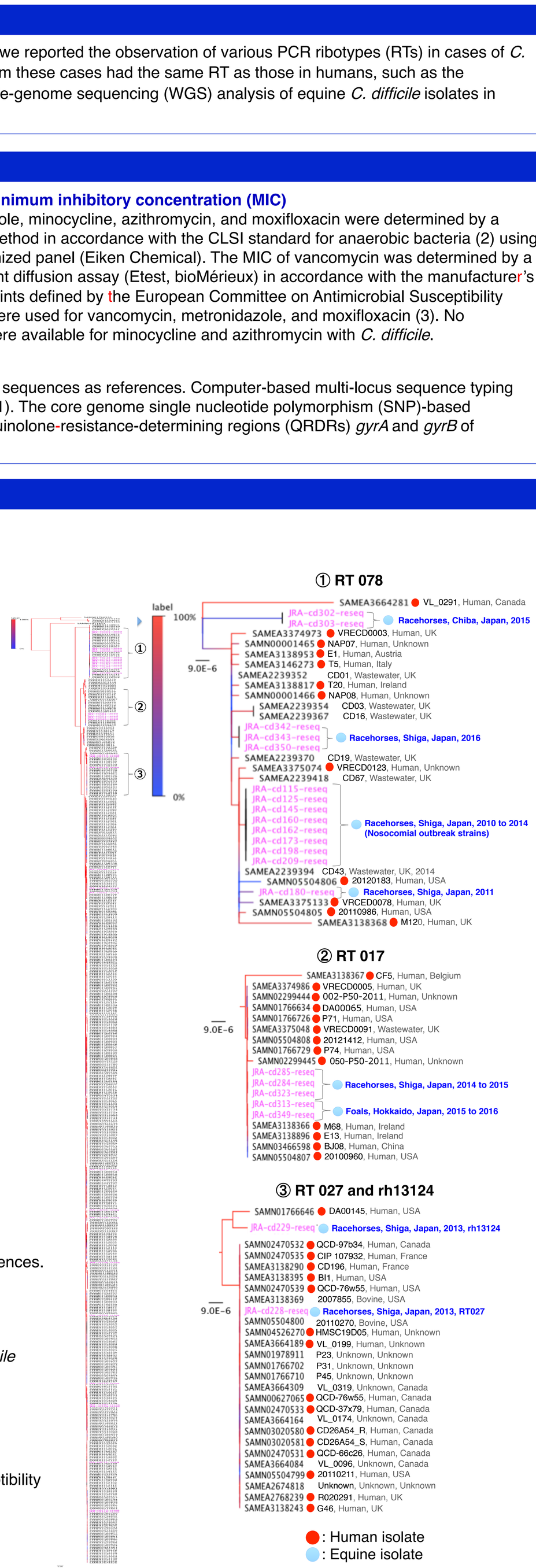

Figure 1. Core-genome SNP-based phylogenic analysis of representative RTs of the equine isolates in Japan and publicly available *C. difficile* strains.

CONCLUSIONS

- ➢ WGS analysis suggested the presence of a particular sublineage of RT 078 in Japanese horses associated with a nosocomial outbreak in an equine clinic.
- ➢ High genetic relatedness between equine and human isolates suggests the possibility of transmission of *C. difficile* between horses and humans.
- ➢ Some antimicrobial resistance genes detected by WGS, corresponding to phenotype, were detected in some RTs.
- ➢ The sources of *C. difficile* in the Japanese equine population are still unknown. Therefore, continuous observation will be important from the perspective of the "One Health" concept.

ACKNOWLEDGEMENTS

We are deeply grateful to Dr. Itoh (Hidaka Horse Breeders' Association, Japan) and Dr. Hara (Hunabashi Racecourse, Japan) for kindly providing horse fecal samples. We thank Ms. Tezuka and Ms. Yomogida for their technical assistance.

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