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Whole genome sequencing reveals potential spread of *Clostridium difficile* between humans and farm animals in the Netherlands, 2002 to 2011

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Abstract

Farm animals are a potential reservoir for human *Clostridium difficile* infection (CDI), particularly PCR ribotype 078 which is frequently found in animals and humans. Here, whole genome single-nucleotide polymorphism (SNP) analysis was used to study the evolutionary relatedness of *C. difficile* 078 isolated from humans and animals on Dutch pig farms. All sequenced genomes were surveyed for potential antimicrobial resistance determinants and linked to an antimicrobial resistance phenotype. We sequenced the whole genome of 65 *C. difficile* 078 isolates collected between 2002 and 2011 from pigs (n = 19), asymptomatic farmers (n = 15) and hospitalised patients (n = 31) in the Netherlands. The collection included 12 pairs of human and pig isolates from 2011 collected at 12 different pig farms. A mutation rate of 1.1 SNPs per genome per year was determined for *C. difficile* 078. Importantly, we demonstrate that farmers and pigs were colonised with identical (no SNP differences) and nearly identical (less than two SNP differences) *C. difficile* clones. Identical tetracycline and streptomycin resistance determinants were present in human and animal *C. difficile* 078 isolates. Our observation that farmers and pigs share identical *C. difficile* strains suggests transmission between these populations, although we cannot exclude the possibility of transmission from a common environmental source.

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Authors' contributions

CWK, JC, EJK, and TDL designed the study. SD, ECK, and LL collected strains and epidemiological data. CWK, IMJG, and ECK did laboratory work and DNA extraction. DH did whole-genome sequencing. CWK, TRC, AM, and HPB did bioinformatic analysis. CWK, TRC, AM, and TDL analysed data. CWK, TRC, AM, JC, EJK and TDL interpreted data. CWK prepared the figures. CWK, TRC,JC, EJK, and TDL wrote the report.

Conflict of interest

None declared.

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Introduction

In the past decade *Clostridium difficile* has emerged rapidly to become the most common cause of antibiotic-associated diarrhoea in healthcare facilities worldwide. Antibiotic treatment, advanced age and hospitalisation are the major risk factors for developing *C. difficile* infection (CDI) leading to diarrhoea, pseudomembranous colitis or death [1,2]. CDI is increasingly recognised in the community setting [3-6] where exposure to antibiotics is an important risk factor [5], while the use of proton pump inhibitors [4], outpatient healthcare exposure [7], obesity and inflammatory bowel disease (IBD) [8] are potential risk factors. *C. difficile* virulence is primarily mediated by two potent enterotoxins, TcdA and TcdB, which are encoded in a pathogenicity locus (PaLoc) [9-11]. The binary toxin may contribute to the virulence of *C. difficile* as well [12], but its role in CDI is still under debate [13-16]. *C. difficile* produces highly resistant and infectious spores, which can survive in the environment for a long time and facilitate environmental transmission within the healthcare setting [17].

Symptomatic individuals are an important source of *C. difficile* transmission in a hospital setting, and patient isolation and antibiotic stewardship have been proven to be effective infection control measures [18,19]. The role of asymptomatic carriers as donors of transmission may also be significant [20-23], and diverse novel subtypes are continuously introduced in the healthcare system, highlighting a link to a large and diverse community reservoir [24]. Interestingly, *C. difficile* PCR ribotype 078, which is commonly found in the healthcare system of various European countries [25], is more often associated with community-acquired CDI [26]. Notably, this variant is the most common type found in pigs [27-30] and other farm animals [31-33].

Several studies have reported an overlap between *C. difficile* genotypes isolated from humans and animals [27,34-38] using conventional typing methods such as PCR ribotyping, multilocus sequence typing (MLST), and multilocus variable-number tandem repeat analysis (MLVA). However, these methods do not have the discriminatory power to distinguish between closely related strains as is required for transmission tracking. In this study, we used whole genome sequencing and phylogenetic analysis to track single clones in human and animal populations to demonstrate potential interspecies transmission.

Methods

Collection of *Clostridium difficile* isolates

In total, the genomes of 65 isolates designated PCR ribotype 078 were sequenced and analysed. Of these 65 isolates, 34 were derived from healthy humans (n=15) and pigs (n=19) on 19 Dutch pig farms (farm isolates) and 31 from hospitalised patients in various Dutch hospitals. Of the farm isolates, 24 isolates were paired by farm (i.e. 12 pairs of human and pig isolates from 12 farms), whereas the remaining 10 (from three farmers and seven pigs) were not paired. The majority of the farm isolates were collected in 2011 by the Institute for Risk Assessment Sciences of the Utrecht University as part of another study [34]. Thirty-one randomly selected clinical isolates originating from various Dutch hospitals between 2002 and 2011 were obtained from the Dutch National *C. difficile* reference laboratory at Leiden

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University Medical Center. In addition, one PCR ribotype 066 strain was included; this strain was obtained from our Leeds-Leiden/European Centre for Disease Prevention and Control (ECDC) reference strain collection [39]. Details of all sequenced isolates are listed in Table 1, including the European Nucleotide Archive (ENA) sample accession numbers. Two isolates were sequenced in duplicate.

Bacterial culture and genomic DNA preparation

C. difficile was cultured on blood agar plates (BioMérieux, the Netherlands), inoculated into liquid medium (brain–heart infusion (BHI) broth supplemented with yeast extract and cysteine) and grown over night (ca 16 hours) anaerobically at 37 °C. Cells were pelleted, washed with phosphate-buffered saline (PBS), and genomic DNA preparation was performed using a phenol–chloroform extraction as previously described [40].

Whole genome sequencing

Paired-end multiplex libraries were created as previously described [41]. Sequencing was performed on an Illumina HiSeq 2000 platform, with a read length of 100 bp.

In silico MLST

The alleles for the seven housekeeping genes used for *C. difficile* MLST [42] (http://pubmlst.org/cdifficile/), adk, atpA, dxr, glyA, recA, sodA, and tpi, were analysed in silico to determine the sequence type (ST). All sequenced genomes were aligned with the CDM120 genome using the multiple sequence alignment editor Seaview [43], after which each individual MLST allele was analysed for sequence variation.

SNP calling and recombination detection

Illumina sequence data were mapped to the *C. difficile* 078 reference genome, M120, (European Molecular Biology Laboratory (EMBL) accession number: FN665653) as paired-end reads using SMALT software (http://smalt.sourceforge.net/), and SNPs were identified as previously described [41]. A potential confounder within the downstream phylogenetic analysis is the effect of homologous recombination, which has the potential to interfere with the phylogenetic signal within the dataset. To alleviate this problem we used the approach developed by Croucher et al. [40] to identify regions in the genome of each isolate where there was evidence of recombination. We then removed those sites from our alignments used in downstream analyses.

Phylogeny and detection of non-phylogenetic SNPs

Phylogenetic trees were constructed using RAxML [44] with a general time reversible (GTR) model with a gamma correction for among-site rate variation combined with 100 random bootstrap replicates (default). Finally, metadata (source, year of isolation, geographical location) was transferred to the reconstructed tree.

Mutation rate estimation

The mutation rate across the population was estimated using the Bayesian evolutionary analysis sampling trees (BEAST) software v1.7.5 [45]. BEAST operates by utilising an

explicit model of evolution to compute the mutation rate on each branch of a phylogenetic tree. This enables the translation of evolutionary time into calendar units: days or years. In order to ensure that the dataset was converging consistently, three independent Markov chain Monte Carlo (MCMC) chains were run, each of 100,000,000 states. From these, we removed an initial 10% as a burn-in (10,000,000 states) for each chain and joined the chains using LogCombiner (part of the BEAST suite), taking a sample every 10,000 states.

Genome-wide scan for antimicrobial resistance determinants

De novo assembly was performed for each sequenced genome using the Velvet assembler [46]. The assembled contigs were then ordered against the reference genome M120 using ABACAS [47], which was required for downstream analysis using Artemis Comparison Tool (ACT) [48]. The ordered contigs were used to perform BLAST homology searches for transposons and antimicrobial resistance determinants. The results of this analysis and the discovery of novel potential transposons were visualised using ACT [48]. In addition, the presence of antimicrobial resistance determinants located on the identified transposons were confirmed using the ResFinder 2.1 server [49], with an 98% threshold for identity.

Antibiotic resistance

The minimum inhibitory concentration (MIC) for tetracycline was determined using E-test (BioMérieux, the Netherlands) on *Brucella* plates (Mediaproducts BV, the Netherlands) under anaerobic conditions at 37 °C. Streptomycin resistance was tested by disk diffusion method, using Sensi-Neotabs 500 µg disks (Rosco, Denmark). Results were interpreted using the tetracycline breakpoints provided by the Clinical and Laboratory Standards Institute (CLSI) [50] and streptomycin breakpoints from Corver et al. [51].

Results

Mutation rate of *Clostridium difficile* 078 from the Netherlands

We performed whole genome sequencing on 65 *C. difficile* 078 strains isolated between 2002 and 2011 from various sources (animal or human) and locations in the Netherlands (Figure 1A; Table 1). The human isolates (n = 46) were obtained either from hospitalised patients suffering from CDI (n = 31) or from asymptomatic colonised humans working on Dutch pig farms (n = 15). *C. difficile* 078 was also isolated from asymptomatic pigs (n = 19). In total, 12 pairs of pig/farmer isolates were included, collected at the same time from the same farms where the sampled farmers resided and worked.

We initially compared the genotypes of the *C. difficile* 078 isolates with MLST, the traditional gold standard for epidemiological typing of bacterial pathogens. MLST analysis was done using the DNA sequences of seven housekeeping genes [42], which were extracted from the whole-genome dataset. The concatenated sequence length of the MLST loci (3,501 nt) represents ca 0.09% of the whole genome. Our results demonstrated that all of the *C. difficile* 078 isolates belonged to ST11, and did therefore not provide a degree of resolution that could be used to track and understand the spread of this organism (data not shown).

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To increase the discriminatory power of the analysis, we mapped the whole genome data for each sequenced isolate to the *C. difficile* 078 reference genome M120 [52] and identified all SNPs. Using this approach we identified 3,927 SNPs within the non-repetitive genome (95.2% of the entire genome). Of these, 3,153 SNPs were identified as acquired through horizontal gene transfer or homologous recombination. These SNPs were removed as they disrupt the true phylogeny, leaving a clonal frame of 774 phylogenetically informative SNPs for further downstream analysis. Of these, 373 SNPs were found only in the *C. difficile* 066 isolate (ST11), a close relative of *C. difficile* 078 [39], which was used to root the phylogenetic tree. A population-specific mutation rate of *C. difficile* 078 was estimated, using the isolation dates of our sequenced samples for calibrating the time scale of the phylogenetic tree. Based on our collection, the mutation rate for the *C. difficile* 078 lineage was estimated to be 2.72×10^{-7} substitutions per site per year (95% confidence interval (CI): 1.43×10^{-7}–3.99×10^{-7}) which is equivalent to 1.1 SNP per genome per year (95% CI: 0.6–1.6) when multiplied by the number of sites present in the *C. difficile* 078 genome. This mutation rate is comparable to published estimates for *C. difficile* 027 [53] and genomes obtained from a selection of 24 distinct STs [54].

Identical genotypes in humans and farm animals—In order to study potential transmission of *C. difficile* 078 between farm animals and humans, we compared 12 pairs of farmer and pig strains by whole genome SNP typing (Table 2). Interestingly, three farmer/pig pairs, collected at three farms located in Heino, Aarle-Rixtel and Moergestel, shared identical genotypes, i.e. had no SNP differences (Table 2). In addition, two pairs collected at farms in Hardenberg and Houten were separated by only one SNP difference. In all probability, one SNP difference is indicative of a very recent potential transmission event (less than one year earlier). Consequently, using one SNP difference as a threshold for defining suspected transmission on farms, the number of potential transmission events between farmers and animals increased to five, representing five of the 12 sequenced farmer/pig pairs. Of the remaining seven paired samples, only two differed more than 10 SNPs, whereas five had three (n = 3), four (n = 1) or seven (n = 1) SNP differences. The paired animal and human samples with only three to four SNP differences could suggest that a potential transmission event occurred a few years before, and from that moment, the bacterium evolved separately inside different hosts. The paired isolates with more than 10 SNPs difference were genetically so diverse that direct transmission was ruled out.

Population structure of *Clostridium difficile* 078 in the Netherlands

To study the closely related paired farm isolates in a broader evolutionary context, we compared the 12 pairs with 41 additional *C. difficile* samples that were epidemiologically unrelated to the farm isolates and collected over a longer period of time. These 41 samples included 10 individual (i.e. unpaired) farm isolates (from three farmers and seven pigs) collected between 2009 and 2011, and 31 independent (i.e. non-outbreak) clinical isolates obtained from hospitalised patients suffering from CDI collected at various Dutch hospitals between 2002 and 2011. According to the definitions described by Kuijper et al. [55], the majority of these clinical isolates (n = 23) were defined as healthcare-associated cases, while two cases were defined as community-associated; for six clinical isolates the onset was unknown (Table 1).

A maximum likelihood phylogeny was generated using the 401 phylogenetic SNPs identified in the genomes of the 65 sequenced isolates (Figure 1B). In total, 61 distinct SNP genotypes were observed among the 65 *C. difficile* 078 isolates. Two isolates (Oirschot ’11 and Leiden ‘06) at the periphery of the phylogenetic tree differed by 49 SNPs, which gave an indication of the extent of variation present in the phylogeny. Interestingly, the inferred phylogeny of Dutch *C. difficile* 078 revealed a general lack of clustering related to strain source (i.e. swine, farmer or clinical), as demonstrated by the mingling of strain sources in the phylogenetic tree (Figure 1B). Isolates from the same source group did not form distinct clusters, while several distinct heterogeneous groups were observed that included isolates obtained from diverse sources. This was especially apparent in the cluster consisting of a clinical isolate (Breda ‘08), a pig isolate (R’donksv.’11) and two farmer isolates (Oirschot ’11 and R’donksv.’11) that were all collected in the same region (Noord Brabant) of the Netherlands (Figure 2). Interestingly, only four SNP differences separated the clinical isolate (Breda ’08) from the nearest farm isolate (R’donksv.’11). Given the three year window in which these isolates were collected and the estimated mutation rate of 1.1 SNP difference per genome per year (95% CI: 0.6–1.6), one would expect to observe two to four SNP differences (prediction interval: 1.8–4.8) between these isolates in case of transmission during this time. Therefore, the observed four SNP difference in this cluster suggests a possible transmission link between farm and clinical isolates.

The phylogenetic tree also demonstrated a general lack of geographic clustering (Figure 1B). This is particularly evident for the isolates from Leiden and Zwolle that were dispersed throughout the phylogeny. This observation suggested that related *C. difficile* 078 strains were widely distributed across the country and were frequently transmitted between locations. Interestingly, the analysis revealed two farmers with no obvious epidemiological link that were colonised with identical *C. difficile* 078 isolates (Figure 1B; green box). The farms were located at Lierop and Ulft (ca 100 km apart), emphasising the lack of geographic signal in these results.

Tetracycline and streptomycin resistance determinants are shared between *Clostridium difficile* 078 strains from humans and pig

C. difficile genomes carry a broad array of mobile genetic elements that are not included in our phylogenetic SNP analysis but often encode clinically relevant phenotypes such as antimicrobial resistance (AMR). We assembled and analysed the *C. difficile* 078 genomes to identify potential mobile elements containing AMR determinants and then mapped these onto the phylogenetic tree. We observed the presence of a mobile element with high homology (92.7%) to a previously described transposon Tn*6190* [51] and a novel potential transposon that was designated as Tn*6235* in this study (data not shown). These transposons potentially confer resistance to various antibiotics, including tetracycline, and each transposon grouped into distinct phylogenetic clusters (Figure 3).

Mobile element Tn*6190*, harbouring tetracycline resistance determinant tetM (EMBL accession number: EU182585.1; 98.9% identity), was present in 24 of the sequenced genomes that were obtained from diverse hosts. The majority of these 24 genomes grouped together in a monophylogenetic cluster (Figure 3; orange dots). Tetracycline susceptibility

testing confirmed that the presence of tetM correlated with tetracycline resistance (Figure 3 orange branches; Table 3). The novel mobile element Tn*6235* was present in its full length (ca 40 kb; 100% homology) in 10 sequenced *C. difficile* 078 genomes that formed a distinct monophylogenetic cluster with strains from various sources (Figure 3; purple dots). Blast homology searches of this genomic region revealed an open reading frame with homology (100% identity) to a putative aminoglycoside 3′-phosphotransferase aphA1 (M26832) which may confer streptomycin resistance. Streptomycin susceptibility testing confirmed that all 10 isolates present in the monophylogenetic cluster were streptomycin-resistant (Figure 3 purple branches; Table 3).

Discussion

We used whole genome sequencing and phylogenetic SNP analysis to study the overlap of *C. difficile* 078 genotypes in animals and humans. In three cases, Dutch farmers were colonised with identical *C. difficile* 078 clones as pigs kept on the same farms. We have also shown that the presence of clonal strains in pigs and farmers was common, as demonstrated by the number of farmer/pig pairs (five of 12) where clonality (defined as ≤ 1 SNP difference) was observed.

The clonal *C. difficile* 078 strains in farmers and farm animals that were identified indicate that interspecies transmission has occurred, although we cannot exclude the possibility that they shared a common (environmental) exposure source, e.g. acquisition of spores from a shared common environmental source. However, we believe that direct transmission is plausible. Firstly, the faecal–oral route is the main route of *C. difficile* transmission, and farmers have a high probability of exposure to pig faeces. Secondly, genomes with zero SNP differences were isolated from farmers and pigs. If acquisition of identical *C. difficile* strains in humans and animals was a result of transmission from a common source, then either it must have been a very recent environmental transmission event or it did not evolve inside either host after the exposure. Finally, the possibility of an intermediate host can be excluded for clonal cases because circulation via an intermediate host for a certain period is likely to result in SNP differences. If the cases of clonal *C. difficile* 078 strains in farmers and farm animals are indeed a result of direct interspecies transmission, it would be informative to know the direction of this transmission.

The faecal–oral route of acquisition makes it logical for the direction to be from pig to human. In addition, the high carriage rates of *C. difficile* among farmers [34,56] also suggest movement from pigs to farmers. This was further supported by the identical antibiotic resistance determinants (tetracycline and streptomycin) shared between animal and human strains, an observation that is in line with previous studies [35,57,58]. The independent insertion of Tn*6235* or Tn*6190* at the same locations in the *C. difficile* 078 genomes (data not shown) in combination with phylogenetic clustering of these isolates, suggest that Tn*6235* and Tn*6190* were introduced once in a progenitor genome that has since then spread in both human and animal hosts. Interestingly, tetracycline is not frequently used in the Dutch healthcare system, whereas it is still the preferred pharmacotherapeutic group for the veterinary industry in the Netherlands [59]. This suggests that tetracycline resistance could be arising in *C. difficile* isolates from pigs and passed on to the human population. Future,

more systematic studies should provide more direct evidence for the direction of transmission.

In addition to the contribution of farm animals as a reservoir for human CDI, we want to emphasise that more than half (58%) of our sequenced farmer/pig pairs were not clonal. Two of the twelve pairs had a SNP difference above 10 SNPs. This suggests that exposure to multiple sources other than farm animals may be responsible for colonisation of the farmers and their pigs. *C. difficile* can be found almost anywhere in the environment (soil, water, and potentially food) making it hard to pinpoint which alternative reservoirs are significant contributors to the spread of *C. difficile* 078 in the community. Currently, several potential (environmental) vectors of transmission have been identified, including but not limited to birds, insects, pets and rodents such as rats and mice [60-64]. Our analysis also revealed two farmers who were not geographically linked but were colonised with identical *C. difficile* 078 isolates. These had been isolated ca 100 km apart from each other, which could suggest exposure to a common environmental source. Another possible explanation could be transport of pigs between the two involved farms that resulted in indirect transmission.

We further analysed the farm isolates in a broader context of clinical isolates with no obvious epidemiological links to the farms. Our analysis demonstrated that all sources, farmer, pig and clinical, were distributed throughout the entire phylogenetic tree and no single clusters per source were identified. These observations are in line with previous research on mixed human and animal *C. difficile* populations [35,65]. Both studies showed that animal isolates did not constitute a distinct lineage from human isolates. A possible explanation for this observation is that *C. difficile* 078 strains may have frequently been transmitted between sources, rather than persisting exclusively in one host. Consequently, the clonal strains in farmers and farm animals we identified may be part of a larger network that could have links with the healthcare system. The heterogeneous phylogenetic cluster with limited SNP diversity shown in Figure 2 is an example of potentially linked clinical and farm isolates. Additional patient data for the clinical isolate Breda ’08 showed that, although symptoms started five days before hospitalisation (suspected community onset), the patient was living in a long-term healthcare facility and therefore constituted a healthcare-associated case.

The strength of this study is that we applied for the first time the highly discriminatory method whole genome SNP typing to study the relatedness of *C. difficile* 078 isolates obtained from farmers and farm animals. A limitation of this study is the small number of clinical samples that were community-associated; such samples may have allowed us to demonstrate more links between farm animals, farmers and the wider community. In addition, the bacterial strain cohort was restricted to isolates obtained in one country, the Netherlands.

The recent trends in epidemiological data show that *C. difficile* 078 is an important type found in the Dutch healthcare system and its prevalence has remained stable between 2009 and 2013 (data not shown). Besides symptomatic patients, other sources play a major role in the spread of *C. difficile* within the healthcare system, for instance asymptomatic carriers visiting a healthcare facility [24]. Asymptomatic carriage can be common among

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hospitalised patients [20,21], although future large studies are needed to determine the precise scale of onward transmission by these carriers. The reservoirs from which these carriers in the community are colonised remain to be elucidated. Importantly, it is becoming clear now that the community reservoir for human CDI is much more diverse and larger than previously expected [24,66]. Here, we demonstrate that transmission from pigs to farmers is one of the potential routes by which *C. difficile* is entering the human population, and that these isolates also carry antimicrobial resistance determinants that might be a result of selection in response to antibiotic exposure in pigs.

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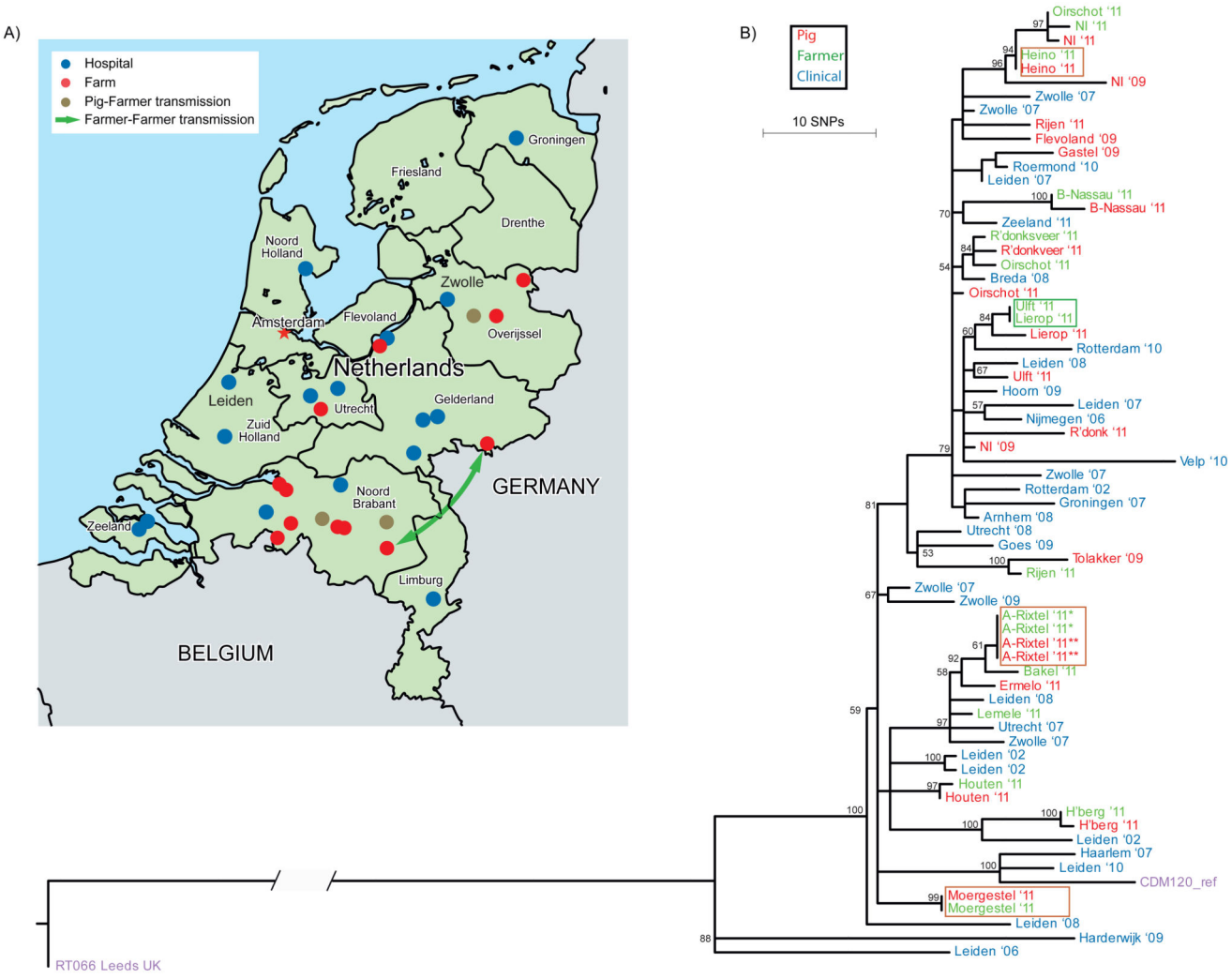

Figure 1. Transmission events and phylogeny of *Clostridium difficile* 078, the Netherlands 2002–11 (n=65)

A. Distribution of Dutch hospitals and pig farms included in this study. Only pig farms with a known location were plotted. Blue dots represent the hospitals (n = 16) where isolates from hospitalised patients were obtained, red dots represent pig farms (n = 12) where isolates from farmers and pigs were obtained. Brown dots represent the pig farms where pigs and farmers had identical C. difficile isolates. The green arrow indicates a potential (long-range) transmission event between two farms.

B. Phylogenetic tree revealing likely transmission between pigs and humans. Shown is the reconstructed phylogenetic tree based on 774 core genome single-nucleotide polymorphisms (SNPs). Samples are colour-coded according to their source: pig (red), farmer (green) and clinical isolate (blue). Identical genotypes with an epidemiological link (i.e. same location/ farm) are marked with brown boxes. Long-range transmission events (i.e. different locations) are marked with a green box. The tip labels are coded with the city name followed by two numbers that represent year of isolation ('08 △ 2008). The CDM120 genome (purple) is used for the reference-based mapping, RT066 (purple) is used as an out-group to

root the tree. The scale indicates the branch length that correspond to 10 SNP differences. The numbers for the internal nodes show the support from 100 non-parametric bootstraps of a maximum likelihood reconstruction (only bootstrap values > 50 are shown).

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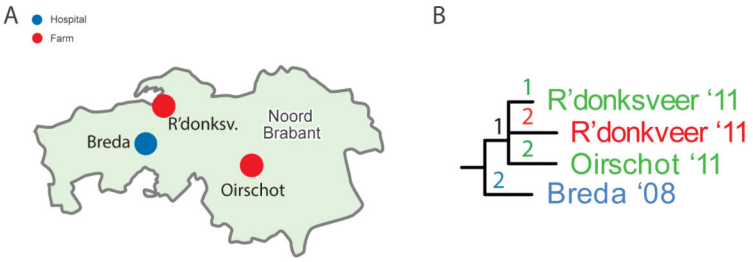

Figure 2. Phylogenetic cluster showing relatedness of *Clostridium difficile* clinical, pig and farmer isolates, the Netherlands, 2008–11 (n=4)

A. Geographical map showing the location of the isolates present in the phylogenetic cluster shown in panel B. Blue dot represents a hospital (Breda), red dots represent the two pig farms (R'donksv. and Oirschot).

B. Zoom-in on a phylogenetic cluster containing highly related isolates from different sources (swine, farmer and clinical isolates). The numbers on the tree branches represent the number of single-nucleotide polymorphisms differences in the cluster, the tip labels are coded with city (Breda) followed by two numbers that represent year of isolation ('08 △ 2008).

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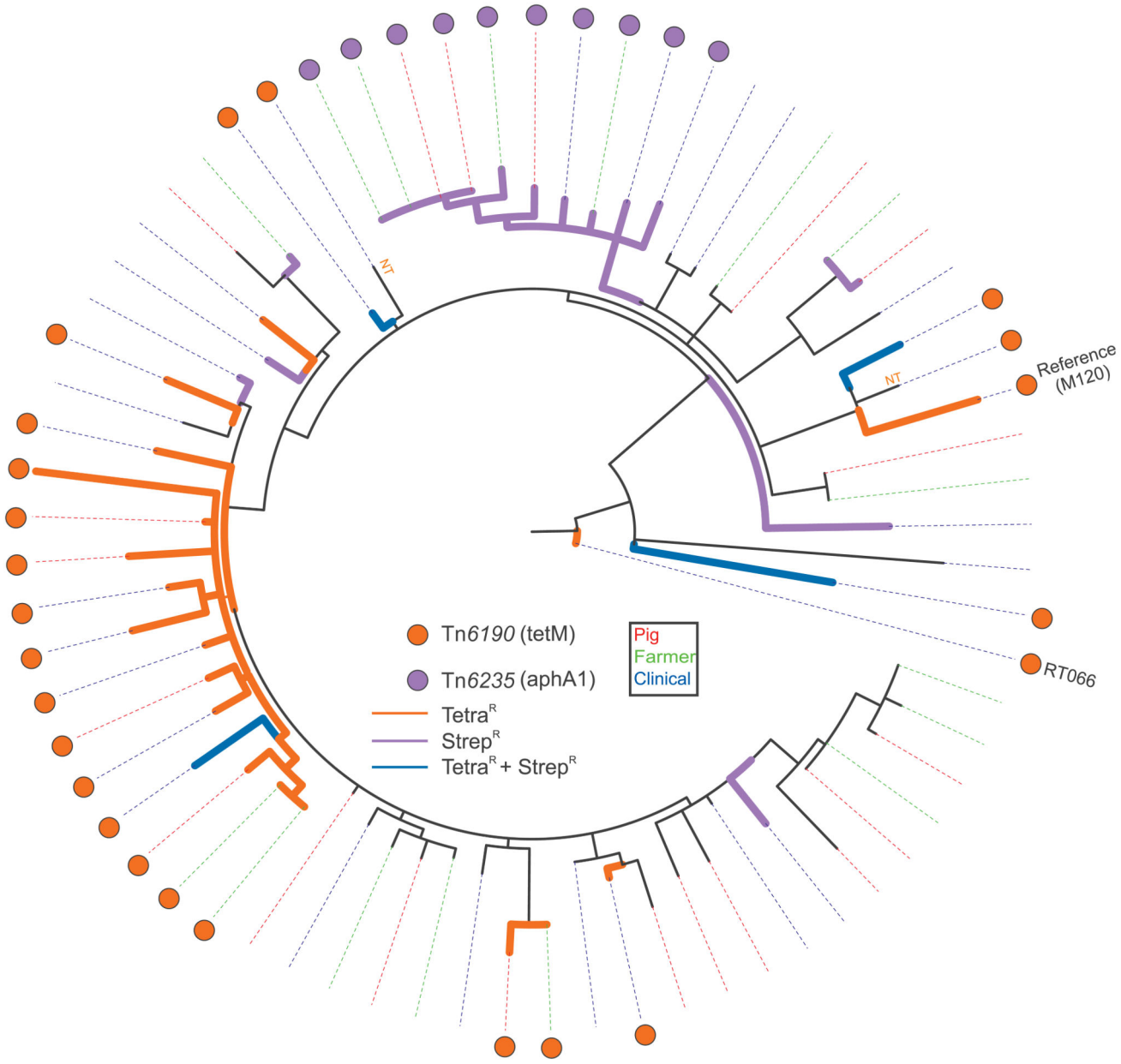

Figure 3. Phylogeny of *Clostridium difficile* 078 isolates showing the presence of antimicrobial resistance determinants, the Netherlands, 2002–11 (n=65[a])

NT = not phenotypically tested.

Circular representation of the C. difficile 078 phylogeny with coloured dots representing the distribution of antimicrobial resistance (AMR) determinants. The legend shows the identified transposons together with the AMR determinants (between brackets) located on the transposon. The coloured dotted lines represent the source of the respective isolates (swine, farmer and clinical isolate). The presence of Tn6190 (tetM) is associated with tetracycline resistance; 078 isolates phenotypically tested as tetracycline-resistant are indicated with orange tree branches, streptomycin-resistant isolates are indicated with purple

tree branches, isolates resistant to both tetracycline and streptomycin are indicated with blue tree branches.

[a] Two isolates were sequenced in duplicate. Ne RT 066 sequence was included as root sequence. In total, 68 sequences are shown.

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Table 1A

Clostridium difficile type 078 isolates used in this study, the Netherlands, 2002–11 (n=65)

R_L#T[a]	Year	City	RT	Isolate	Source	Related isolates	Association	ENA ID[b]
8080_2#24	2006	Leiden	078	6072310	Clinic	Non-outbreak	Healthcare	ERS138026
8080_2#25	2006	Nijmegen	078	6086336	Clinic	Non-outbreak	Healthcare	ERS138027
8080_2#26	2007	Leiden	078	7001233	Clinic	Non-outbreak	Healthcare	ERS138028
8080_2#27	2007	Groningen	078	7004578	Clinic	Non-outbreak	Unknown	ERS138029
8080_2#28	2007	Utrecht	078	7005405	Clinic	Non-outbreak	Unknown	ERS138030
8080_2#29	2007	Zwolle	078	7021455	Clinic	Non-outbreak	Healthcare	ERS138031
8080_2#30	2007	Zwolle	078	7044912	Clinic	Non-outbreak	Community	ERS138032
8080_2#31	2007	Zwolle	078	7066827	Clinic	Non-outbreak	Community	ERS138033
8080_2#32	2007	Zwolle	078	7071308	Clinic	Non-outbreak	Healthcare	ERS138034
8080_2#33	2007	Zwolle	078	7086074	Clinic	Non-outbreak	Healthcare	ERS138035
8080_2#34	2007	Leiden	078	7091952	Clinic	Non-outbreak	Healthcare	ERS138036
8080_2#35	2008	Leiden	078	8011061	Clinic	Non-outbreak	Healthcare	ERS138037
8080_2#36	2008	Utrecht	078	8013820	Clinic	Non-outbreak	Healthcare	ERS138038
8080_2#37	2008	Leiden	078	8051728	Clinic	Non-outbreak	Healthcare	ERS138039
8080_2#38	2008	Leiden	078	8055344	Clinic	Non-outbreak	Healthcare	ERS138040
11250_1#22	2008	Arnhem	078	8056692	Clinic	Non-outbreak	Unknown	ERS362924
8080_2#40	2008	Breda	078	8091554	Clinic	Non-outbreak	Healthcare	ERS138042
8080_2#41	2009	Harderwijk	078	9012668	Clinic	Non-outbreak	Healthcare	ERS138043
8080_2#42	2009	Goes	078	9019497	Clinic	Non-outbreak	Unknown	ERS138044
8080_2#43	2009	Hoorn	078	9077637	Clinic	Non-outbreak	Healthcare	ERS138045
8080_2#44	2010	Roermond	078	10005075	Clinic	Non-outbreak	Healthcare	ERS138046
8080_2#45	2010	Rotterdam	078	10015222	Clinic	Non-outbreak	Healthcare	ERS138047
8080_2#46	2010	Velp	078	10080193	Clinic	Non-outbreak	Healthcare	ERS138048
8080_2#47	2011	Zeeland	078	11012929	Clinic	Non-outbreak	Healthcare	ERS138049
8080_2#49	2009	Zwolle	078	1103	Clinic	Non-outbreak	Unknown	ERS138051
8080_2#58	NI	Leeds	066	066 (root)[c]	Clinic	Reference collection	Unknown	ERS138052
8080_2#61	2002	Rotterdam	078	126065	Clinic	Non-outbreak	Unknown	ERS138053
8080_2#62	2002	Leiden	078	126819	Clinic	Non-outbreak	Healthcare	ERS138054
8080_2#63	2002	Leiden	078	126938	Clinic	Non-outbreak	Healthcare	ERS138055
8080_2#64	2002	Leiden	078	129820	Clinic	Non-outbreak	Healthcare	ERS138056
8080_2#71	2010	Leiden	078	53737	Clinic	Non-outbreak	Healthcare	ERS138060
8080_2#72	2007	Haarlem	078	47337	Clinic	Non-outbreak	Healthcare	ERS138063
8080_2#50	2009	Gastel	078	P29	Pig	Un-paired	Farm	ERS138064
8080_2#51	2009	NI	078	P60	Pig	Un-paired	Farm	ERS138065
8080_2#52	2009	Flevoland	078	P27	Pig	Un-paired	Farm	ERS138066
8080_2#53	2009	NI	078	P70	Pig	Un-paired	Farm	ERS138069

R_L#T[a]	Year	City	RT	Isolate	Source	Related isolates	Association	ENA ID[b]
8080_2#54	2009	Tolakker	078	P52	Pig	Un-paired	Farm	ERS138070
8080_2#67	2011	Aarle-Rixtel	078	H205[d]	Farmer	Pair 1	Farm	ERS138073
8080_2#68	2011	Aarle-Rixtel	078	B37_2[e]	Pig	Pair 1	Farm	ERS138074
9221_6#55	2011	NI	078	H102	Farmer	Pair 12	Farm	ERS199786
9221_6#56	2011	Raamsdonksveer	078	B31_3	Pig	Pair 9	Farm	ERS199787
9221_6#57	2011	Heino	078	B17_3	Pig	Pair 4	Farm	ERS199788
9221_6#58	2011	Ulft	078	H121	Farmer	Pair 11	Farm	ERS199789
9221_6#59	2011	Rijen	078	B27_7	Pig	Pair 10	Farm	ERS199790
9221_6#60	2011	Baarle-Nassau	078	H230	Farmer	Pair 2	Farm	ERS199791
9221_6#61	2011	Oirschot	078	H189	Farmer	Pair 8	Farm	ERS199792
9221_6#62	2011	Lierop	078	B23_6	Pig	Pair 6	Farm	ERS199793

NI: not identified; RT: ribotyope.

[a] R_L#T, run, lane and tag number.

[b] European Nucleotide Archive sample submission number.

[c] Included as root sequence.

[d] Sequenced in duplicate.

[e] Sequenced in duplicate.

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Table 1B

Clostridium difficile type 078 isolates used in this study, the Netherlands, 2002–11 (n=65)

R_L#T[a]	Year	City	RT	Isolate	Source	Related isolates	Association	ENA ID[b]
9221_6#63	2011	Aarle-Rixtel	078	H205[d]	Farmer	Duplicate	Farm	ERS199794
9221_6#64	2011	Hardenberg	078	B15_1	Pig	Pair 3	Farm	ERS199795
9221_6#65	2011	Oirschot	078	H21	Farmer	Un-paired	Farm	ERS199796
9221_6#66	2011	Oirschot	078	B30_5	Pig	Pair 8	Farm	ERS199797
9221_6#67	2011	Rijen	078	H122	Farmer	Pair 10	Farm	ERS199798
9221_6#68	2011	Hardenberg	078	H95	Farmer	Pair 3	Farm	ERS199799
9221_6#69	2011	Raamsdonk	078	B28_1	Pig	Un-paired	Farm	ERS199800
9221_6#70	2011	Bakel	078	H214	Farmer	Un-paired	Farm	ERS199801
9221_6#71	2011	Raamsdonksveer	078	H158	Farmer	Pair 9	Farm	ERS199802
9221_6#72	2011	Heino	078	H88	Farmer	Pair 4	Farm	ERS199803
9221_6#73	2011	Lemele	078	H111	Farmer	Un-paired	Farm	ERS199804
9221_6#74	2011	Baarle-Nassau	078	B39_4	Pig	Pair 2	Farm	ERS199805
9221_6#75	2011	Moergestel	078	B4_2	Pig	Pair 7	Farm	ERS199806
9221_6#76	2011	NI	078	B20_1	Pig	Pair 12	Farm	ERS199807
9221_6#77	2011	Moergestel	078	H16	Farmer	Pair 7	Farm	ERS199808
9221_6#78	2011	Aarle-Rixtel	078	B37_3[e]	Pig	Duplicate	Farm	ERS199809
9221_6#79	2011	Ermelo	078	B1_5	Pig	Un-paired	Farm	ERS199810
9221_6#80	2011	Lierop	078	H170	Farmer	Pair 6	Farm	ERS199811
9221_6#81	2011	Ulft	078	B22_6	Pig	Pair 11	Farm	ERS199812
9221_6#82	2011	Houten	078	B29_10	Pig	Pair 5	Farm	ERS199813
9221_6#83	2011	Houten	078	H141	Farmer	Pair 5	Farm	ERS199814

NI: not identified; RT: ribotype.

[a] R_L#T, run, lane and tag number.

[b] European Nucleotide Archive sample submission number.

[c] Included as root sequence.

[d] Sequenced in duplicate.

[e] Sequenced in duplicate.

Table 2

Single nucleotide polymorphism differences between paired farm isolates of *Clostridium difficile* 078, the Netherlands 2009–11 (n=24)

Pair number	R_L#T[a]	Location of farm	RT	Source	ID	SNP differences
1	8080_268_6#78	Aarle-Rixtel	078	Pig	B37_2	0
	8080_2#67		078	Farmer	H205	
2	9221_6#74	Baarle-Nassau	078	Pig	B39_4	3
	9221_6#60		078	Farmer	H230	
3	9221_6#64	Hardenberg	078	Pig	B15_1	1
	9221_6#68		078	Farmer	H95	
4	9221_6#57	Heino	078	Pig	B17_3	0
	9221_6#72		078	Farmer	H88	
5	9221_6#82	Houten	078	Pig	B29_10	1
	9221_6#83		078	Farmer	H141	
6	9221_6#62	Lierop	078	Pig	B23_6	4
	9221_6#80		078	Farmer	H170	
7	9221_6#75	Moergestel	078	Pig	B4_2	0
	9221_6#77		078	Farmer	H16	
8	9221_6#66	Oirschot	078	Pig	B30_5	10
	9221_6#61		078	Farmer	H189	
9	9221_6#56	Raamsdonksveer	078	Pig	B31_3	3
	9221_6#71		078	Farmer	H158	
10	9221_6#59	Rijen	078	Pig	B27_7	19
	9221_6#67		078	Farmer	H122	
11	9221_6#81	Ulft	078	Pig	B22_6	7
	9221_6#58		078	Farmer	H121	
12	9221_6#76	NI	078	Pig	B20_1	3
	9221_6#55		078	Farmer	H102	

ID: sample identifier. RT: ribotyope.

[a] R_L#T, run, lane and tag number.

Table 3
Results of Antimicrobial susceptibility testing

Isolate	Source	Streptomycin	Tn*6235*	Tetracycline	Tn*6190*
6072310	Clinic		Absent		Present
6086336	Clinic		Absent		Present
7001233	Clinic	NT	Absent		Absent
7004578	Clinic		Absent		Present
7005405	Clinic		Present		Absent
7021455	Clinic		Absent		Present
7044912	Clinic		Present		Absent
7066827	Clinic		Absent		Present
7071308	Clinic		Absent		Absent
7086074	Clinic		Absent		Absent
7091952	Clinic		Absent		Present
8011061	Clinic		Absent		Absent
8013820	Clinic		Absent		Absent
8051728	Clinic		Absent		Present
8055344	Clinic		Present		Absent
8056692	Clinic		Absent		Absent
8091554	Clinic		Absent		Absent
9012668	Clinic		Absent		Absent
9019497	Clinic		Absent		Absent
9077637	Clinic		Absent		Present
10005075	Clinic		Absent		Present
10015222	Clinic		Absent		Present
10080193	Clinic		Absent		Present
11012929	Clinic		Absent		Absent
1103	Clinic	NT	Absent	NT	Present
P29	Pig	NT	Absent		Absent
P60	Pig	NT	Absent		Present
P27	Pig		Absent		Absent
P70	Pig	NT	Absent		Absent
P52	Pig	NT	Absent		Absent
RT066	Clinic		Absent		Present
126065	Clinic	NT	Absent	NT	Absent
126819	Clinic	NT	Absent	NT	Absent
126938	Clinic	NT	Absent	NT	Absent
129280	Clinic		Absent		Absent
H205	Farmer		Present		Absent
B37.3	Pig		Present		Absent

Isolate	Source	Streptomycin	Tn*6235*	Tetracycline	Tn*6190*
53737	Clinic	NT	Absent	NT	Present
47337	Clinic		Absent		Present
H102	Farmer		Absent		Absent
B31.3	Pig		Absent		Absent
B17.3	Pig		Absent		Absent
H121	Farmer		Absent		Present
B27.7	Pig		Absent		Absent
H230	Farmer		Absent		Present
H189	Farmer		Absent		Absent
B23.6	Pig		Absent		Present
H205	Farmer		Present		Absent
B15.1	Pig		Absent		Absent
H21	Farmer		Absent		Absent
B30.5	Pig		Absent		Absent
H122	Farmer		Absent		Absent
H95	Pig		Absent		Absent
B28.1	Pig		Absent		Present
H214	Farmer		Present		Absent
H158	Farmer		Absent		Absent
H88	Farmer		Absent		Absent
H111	Farmer		Present		Absent
B39.4	Pig		Absent		Present
B4.2	Pig		Absent		Absent
B20.1	Pig		Absent		Absent
H16	Farmer		Absent		Absent
B37.3	Pig		Present		Absent
B1.5	Pig		Present		Absent
H170	Farmer		Absent		Present
B22.6	Pig		Absent		Present
B29.10	Pig		Absent		Absent
H141	Farmer		Absent		Absent

NT: not available for testing; shown are the distribution of the mobile elements Tn*6190* and Tn*6235* among the 078 genomes.

Green: sensitive (S); orange: intermediate (I); red: resistant (R).

Minimum inhibitory concentration cut-off levels used:

Antibiotic	S	I	R
Tetracycline (µg/mL)	‹4	8	≥16
Streptomycin (mm)	≥15	NA	‹15

mm: zone diameter breakpoint in mm; NA: not applicable.