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Whole genome sequencing of toxigenic *Clostridium difficile* in asymptomatic carriers: insights into possible role in transmission

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SUMMARY

Background: Estimates of the prevalence of asymptomatically carried *Clostridium difficile* in elderly patients in long-term care range from 0% to 51%. Asymptomatic carriage is possibly a risk factor for the development of infection, and there is ongoing debate surrounding the role of asymptomatic carriage in transmission.

Aim: To investigate the prevalence of asymptomatic carriage amongst patients residing in intermediate care (bedded) facilities (ICBFs), and to investigate whether asymptomatically carried *C. difficile* strains contribute to nosocomial *C. difficile* infection (CDI).

Methods: Stools were collected from eligible asymptomatic patients in ICBFs, and a subset was also processed from symptomatic patients accessing primary or secondary care outside of ICBFs. All samples were cultured for *C. difficile*, and resulting colonies were processed through whole genome sequencing.

Findings: In total, 151 asymptomatic patients were sampled, 22 of which were positive for *C. difficile* through stool culture, representing a carriage rate of 14.6%. Sequencing of these isolates, alongside 14 *C. difficile* polymerase chain reaction and culture-positive isolates from symptomatic individuals, revealed that all asymptomatic patients were carrying toxigenic *C. difficile*, and these strains were genetically similar to those from symptomatic patients.

Conclusion: This small study of asymptomatic carriage revealed a rectal asymptomatic carriage rate of 14.6% in patients nursed in ICBFs, and a high level of genetic similarity of these strains to those recovered from symptomatic patients. As such, asymptomatic carriers may be important for the transmission of symptomatic CDI, although it is acknowledged that this study was small, and many other factors govern whether *C. difficile* is carried asymptomatically or causes symptoms.

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Introduction

Clostridium difficile infection (CDI) is a major concern in healthcare settings, where it is mostly associated with elderly patients who are at greatest risk due to increased rates of hospital admission, interventions and visits; and increased consumption of (broad-spectrum) antibiotics that disrupt the protective gut microbiome, allowing the *C. difficile* spores to germinate, colonize the gut and produce enterotoxins [1]. Recent hospitalization, previous antibiotic treatment, previous CDI, immunosuppression, use of proton pump inhibitors, surgical interventions, and living in a care facility are additional risk factors for CDI [2]. Infection with *C. difficile* typically manifests as a wide spectrum of illness, ranging from mild diarrhoea to severe complications including pseudomembraneous colitis, toxic megacolon, bowel perforation, sepsis and death [3].

Asymptomatic carriage of toxigenic *C. difficile* is defined as the presence of *C. difficile* in the absence of diarrhoea (or, if present, attributable to a cause other than CDI) or pseudomembraneous colitis [4]. According to the published literature, prevalence rates of asymptomatic carriage vary considerably between patient groups, being highest in healthy neonates and infants (18–90%) [5–7], and lowest in healthy adults in the general population (0–15%) [8–11]. For elderly patients being nursed in long-term care facilities, chronic care or nursing homes, carriage rates range from 0% to 51% [9,12,13], which most likely reflects the method of testing, how the studies were performed, and the presence of recent outbreaks. Rea *et al.* [14] investigated *C. difficile* carriage (toxigenic and non-toxigenic) in elderly subjects using culture, and found that carriage rates varied, with low rates in community patients (1.6%) and higher rates in outpatients (9.5%) and those in short- or long-term care facilities (21%). Furthermore, Nissle *et al.* [2] investigated a series of elderly patients for asymptomatic carriage of *C. difficile* following admission to hospital. Stool samples for 262 consecutive patients were tested for toxigenic *C. difficile* using polymerase chain reaction (PCR), and resulted in a carriage rate on admission of 16.4% for this cohort of patients.

Infection prevention and control (IPC) measures for symptomatic patients with CDI include source isolation (barrier nursing), patient isolation and treatment, and antimicrobial stewardship [15]. To date, only one study [16] has investigated the impact of implementing IPC measures on asymptomatic patients. Longtin *et al.* [16] performed *C. difficile* toxin (*tcdB*) PCR as a screening test on all admissions to a Canadian hospital, and positive patients were isolated. The intervention resulted in a significant reduction in the incidence of CDI ($P < 0.001$) compared with the pre-intervention period. Three further studies confirmed that asymptomatic carriers are important in transmission to other patients [16–18].

With this in mind, the present study was conducted to investigate the prevalence of asymptomatic *C. difficile* carriage amongst elderly patients within inpatient rehabilitation services residing in a number of intermediate care (bedded) facilities (ICBFs) managed by one large community organization. Whole genome sequencing (WGS) was performed on all culture-positive samples (as well as on a subgroup of other samples) to investigate whether there was any evidence to suggest that asymptomatically carried *C. difficile* strains contribute to nosocomial CDI.

Methods

This study was performed by collecting samples from patients residing in a number of ICBFs in Birmingham, UK over a 100-day period. The ICBFs encompassed 18 wards set across seven sites (A–G) (Table I and Table A, see online supplementary material).

All asymptomatic inpatients (nursed within the ICBFs during the study period) were considered for inclusion in the study. Patients were excluded from the study if they met any of the exclusion criteria: aged <65 years, colostomy (or stoma) *in situ*, unexplained diarrhoea, or active (current) or past CDI (within the preceding three months). For exclusion purposes, active CDI was defined as the presence of diarrhoeal symptoms (three or more unformed stools in 24 or fewer consecutive hours) alongside the detection of toxigenic *C. difficile* by PCR (Gene Xpert, Cepheid, Sunnyvale, CA, USA) [17].

Patients were also excluded if it was envisaged that there would be difficulty in producing a faecal sample during the collection period. Ethical approval was granted [REC reference number (14/WM/0096), NRES Ethics Committee (West Midlands – Coventry and Warwickshire)] prior to commencement of the study, and consent was sought prior to sample collection.

Sample collection from asymptomatic patients

A single faecal sample was collected from each patient, and couriered directly to the microbiology department of the local acute hospital for processing via culture. All samples were labelled sequentially, with the prefix 'AS' to denote 'asymptomatic'.

Sample collection from symptomatic patients

To act as a reference, all *C. difficile* PCR positive samples received in the microbiology laboratory at the acute hospital from symptomatic patients in the community (during the 100-day study period), were also cultured for *C. difficile*. These were labelled with the prefix 'S' for 'symptomatic', and the geographical location of the patient at the time of sample collection was recorded.

Clostridium difficile culture and polymerase chain reaction

A systematic evaluation of culture-based methods to recover *C. difficile* from stool samples by Hink *et al.* [18], alongside other examples in the literature [19,20], demonstrated the value of using broth enrichment culture prior to solid culture for recovery. Due to the expected small numbers of organisms in asymptomatic individuals, this method was employed, alongside an ethanol shock stage, which is in-line with the Public Health England UK Standards (SMI B10) [21].

In brief, the broth enrichment culture stage involved adding 200 μg (or 200 μL if liquid) of faeces into Robertson's cooked meat broth (Oxoid, Basingstoke, UK), and incubating at 35–37°C for 48 h. After 48 h, 1 mL of the enriched culture was added to 1 mL of absolute ethanol in a bijou, mixed thoroughly by vortexing, and incubated at room temperature for 30 min.

Following this treatment, two drops (50–75 μL) of the deposit (the lower faecal layer) were inoculated on to solid

Table I
Sites, number of wards, number of asymptomatic patients sampled, and details of the positive results (sample name, ward, day of study) and asymptomatic carriage rate

Site	Number of wards	Number of asymptomatic patients recruited to the study			Number of asymptomatic patients positive for *Clostridium difficile*	Details of the positive result (sample label/ward/day of study)	Asymptomatic carriage rate (%)
		Total number recruited	Number sampled by stool	Number who were recruited but were unable to produce a stool when the ward(s) were visited			
A	1	30	14	16	2	AS_082/1/d14 AS_084/1/d14	14.3
B	1	8	5	3	0	-	-
C	1	22	11	11	2	AS_032/1/d7 AS_050/1/d7	18.2
D	4	36	22	14	5	AS_092/4/d16 AS_094/4/d16 AS_369/1/d99 AS_376/3/d99 AS_386/4/d99	22.7
E	5	121	68	53	11	AS_136/4/d39 AS_142/3/d42 AS_147/3/d42 AS_196/2/d50 AS_204/1/d66 AS_220/1/d74 AS_222/1/d74 AS_231/1/d74 AS_239/1/d74 AS_340/5/d94 AS_359/5/d95	16.2
F	4	28	17	11	0	-	-
G	2	25	14	11	2	AS_298/1/d85 AS_361/1/d95	14.3
Totals	18	270	151	119	22	Asymptomatic carriage rate (%)	14.6

Brazier's *C. difficile* selective agar (Oxoid), and all plates were incubated anaerobically at 35–37°C for 48 h using an anaerobic chamber (Whitley A45 anaerobic workstation). For quality control, spore suspensions of an American Type Culture Collection (ATCC) control strain of *C. difficile* (ATCC9689) were plated on Brazier's agar and incubated as detailed above. This was included in every batch of samples processed by culture.

All faecal samples also underwent PCR testing for tcdB, *cdt* (binary toxin gene) and the *tcdC* deletion at base 117 associated with the ribotype 027 strain using the GeneXpert *C. difficile* BT kit (Cepheid), in accordance with the manufacturer's instructions.

Colony identification

Following culture, suspect colonies from asymptomatic and symptomatic samples were identified through matrix-assisted laser desorption ionization time-of-flight mass spectrometry using the VITEK-MS platform (bioMérieux, Basingstoke, UK), and those positive for *C. difficile* were subcultured on to two Columbia blood agar plates (bioMérieux) and placed back in the incubator for a further three days to encourage sporulation.

Sporulated isolates were prepared for long-term storage in tubes containing 1.5 mL of 2:1 absolute ethanol to physiological saline, which were then placed at −20°C.

DNA extraction

A subset of *C. difficile* positive cultures was subjected to DNA extraction and WGS. These comprised all the positive samples from asymptomatic patients, and a subset of positive samples from symptomatic patients to act as a reference.

DNA was extracted from 48-h-old colonies of *C. difficile* (present on solid agar) using the QuickGene DNA tissue kit (Kurabo, Osaka, Japan) and the QuickGene-Mini80 platform (Fujifilm, Tokyo, Japan) in accordance with the manufacturers' instructions. This kit was chosen as it was recommended by the National Anaerobe Reference Laboratory (personal communication). In brief, a heavy suspension of colonies was prepared in a solution comprising 180 μL of tissue lysis buffer and 20 μL of proteinase K, and incubated for 60 min at 55°C with gentle agitation. The solution was then vortexed and centrifuged (at 8000 × g for 3 min at room temperature), and the supernatant was transferred to a new tube containing lysis buffer and absolute (>99%) ethanol. The lysate was subjected to pressurization and washing as per the QuickGene-Mini80 extraction protocol [22]. This system employs a silica membrane to capture the DNA in the lysate (through binding). Purification is achieved through successive washes with a wash buffer, before the DNA is eluted (unbound from the membrane) into a final volume of 200 μL. DNA yields present in 5 μL of eluate were quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher, Loughborough, England).

Whole genome sequencing and data analyses

Genome sequencing of the *C. difficile* strains was conducted using the Illumina NextSeq 500 system. The DNA of the strains was fragmented, tagged, quantified and finally normalized to equal concentrations according to the Nextera XT protocol [23] using the manufacturer's recommendations. Paired-end sequencing with 150 cycles each end was performed in-house.

The reads from the sequencer were trimmed using trimmomatic version 0.35 [24] using MINLEN as 40 bp and ILLUMINACLIP:NexteraPE-PE.fa:2:30:10. The trimmed reads were assembled using SPAdes version 3.11.1 [25] with the –sensitive parameter. The contigs were annotated using prokka version 1.12 [26] using –usegenus –cdifficile parameters. To determine the identity between the genomes, average nucleotide identity (ANI) was calculated using the ANI/AAI matrix from the Kostas laboratory [27] between the de-novo assembled genomes with *C. difficile* 630 (AM180355) as the reference. Sequence typing of the *C. difficile* strains was performed using the multi-locus sequence typing (MLST) tool version 2.8 designed by Torsten Seeman [28].

To determine single nucleotide polymorphisms (SNPs) between the de-novo assembled *C. difficile* genomes, SNPs were detected using Snippy version 3.1 [29] by recommended parameters. The Snippy-core from Snippy tool was used to determine the core SNPs (i.e. SNPs present in all samples were used for constructing the phylogenetic tree). The tree was viewed using Mega version 7. SNP distances were calculated by taking all of the FASTA alignments between the reference and strains, and calculating the distance using snp-dists version 0.2 [28].

All genomes with >95% identity were used for core and accessory gene identification with Roary version 3.12.0 [30]. The .gff files from prokka annotation were used as input to Roary, with *C. difficile* 630 as the reference strain. Fripan [31] was used for constructing the multi-dimensional scaling (MDS) plot.

Ribotyping

C. difficile PCR ribotyping was also performed on all symptomatic and asymptomatic isolates at the Public Health England reference laboratory (Heartlands Hospital, Birmingham), according to standard protocols.

Results

Prevalence of asymptomatic carriage

In total, 151 stools (collected from 151 patients) were received into the acute hospital microbiology laboratories during the sampling period. A further 119 patients were eligible and consented to take part, but were unable to produce a stool sample on the day(s) when the research nurse was present in the ward (Table I). No patients were excluded based on the exclusion criteria. In terms of the sites and included wards that were sampled, no outbreaks of *C. difficile* had been documented in the six months prior to the start of the study, but acute referring hospitals were known to have experienced outbreaks (although the details of such were not available to the researchers at the time of the study).

There were 22 stool samples (corresponding to 22 patients) positive for *C. difficile* through culture, representing a rectal carriage rate for this population of approximately 14.6%. There was no temporal pattern to the positives (they were found in each month of the 100-day study), but some geographical clustering was seen, with 11 of the 22 positives deriving from several wards contained within Site E. The remaining positives came from four other sites.

Genetic relatedness between the symptomatic and asymptomatic strains

Sequencing was performed on colonies isolated from all 22 samples from asymptomatic patients in the community, and a selection of 14 *C. difficile* PCR and culture-positive isolates from symptomatic individuals (from a total of 83 isolates). These were selected to represent those most likely to be epidemiologically linked to the ICBFs. For example, wards from the ICBFs were included, alongside several geriatric wards from the acute hospital, and local general practitioner surgeries.

The sequences produced a total of 18 Gb of sequence with an average of 3,350,174 reads/sample, and have all been uploaded to the NCBI BioSample database [32], with accession numbers SAMN09979693–SAMN09979727 (SRA accession number PRJNA490071). Approximately 6–7% of reads were discarded after trimming and quality filtering. An ANI assessment was performed on all the sequenced strains [33]. This confirmed that all isolates were *C. difficile* with percentage nucleotide similarity values (to *C. difficile* reference genome 630) of 98–100% for all (Figure A, see online supplementary material). A contamination check was also performed and demonstrated that 100% of the reads were *Clostridia* spp. and that there was no contamination (note: phix was not utilized during sequencing) [Table B(i) and B(ii), see online supplementary material]. Table C (see online supplementary material) gives a summary of the assembly statistics. Two of the symptomatic isolates (S_52 and S_77) were found to contain a mix of *C. difficile* strains, and were therefore excluded from further analysis.

The sequencing data do not support a hypothesis that asymptomatic and symptomatic strains of *C. difficile* form two discrete populations. Pangenome-wide analysis (comparing the whole genomic content between the genomes) shows that there are a large number of core and accessory genes that are shared between the asymptomatic and symptomatic isolates (Figure B, see online supplementary material). Approximately 39% of all the genes were present in all 34 samples (and therefore deemed 'core genes'), with a further 1.6% of the genes classified as 'soft-core' as they were present in 32–33 of the samples. The rest of the sections of the plot ('shell' and 'cloud') comprise the accessory genome, found in fewer than 32 and five samples, respectively. The core and accessory genomes were plotted in an MDS plot (Figure 1). The MDS plot indicates no major differences between the symptomatic and asymptomatic genomes.

When the data are presented as a maximum likelihood phylogenetic tree (plotted based on the similarity of the core genomes between the isolates), several clusters are revealed (Figure 2). These involve either isolates from asymptomatic patients alone (three pairs of samples), or isolates from both asymptomatic and symptomatic patients, and involve a mixture of toxin-positive and -negative strains. The three clusters of isolates from asymptomatic individuals comprise three pairs of samples (marked with an asterisk on the figure), with each pair deriving from the same site and, in some cases, the same ward. For example, AS_220 and AS_222 were collected from Beds 10 and 12, respectively, at Site E on the same date. Similarly, AS_094 and AS_369 both derive from Site D, but different wards, and were collected 83 days apart. Overlaying data on SNPs reveals an SNP difference of 12 nucleotide positions between AS_220 and AS_222, and nine nucleotide positions between AS_094 and AS_369. The SNP data for all isolates are shown in Table D (see online supplementary material). There is a mean average of 9727 SNPs when asymptomatic isolates are compared with each other, 11,072 when symptomatic isolates are compared with each other, and 11,345 when asymptomatic isolates are compared with symptomatic isolates. A SNP distance matrix is shown in Table II for sample pairs where there was a difference up to 50 SNPs.

The four clusters containing isolates from asymptomatic and symptomatic patients vary in size from two to six isolates. Cluster 4 (Figure 2) contains four asymptomatic isolates from Site E: two from Ward 1 (AS_204 and AS_239) and two from Ward 3 (AS_142 and AS_147). There are 18 SNPs between AS_142 (Ward 3) and AS_204 (Ward 1). The cluster also contains one asymptomatic isolate from Site D (AS_092), and a symptomatic isolate (S_51) from a patient who was also nursed at Site E approximately 10 days later. The SNP differences between the asymptomatic isolates in this cluster are small, ranging from 18 (AS_142 compared with AS_204) to 104 (AS_092 compared with AS_147), and there is also a low number of SNP differences between the asymptomatic isolates and the single symptomatic isolate (SNP differences range from 25 to 105) (Table A, see online supplementary material). The other three clusters (1, 2 and 3) contain isolates from a variety of sites. The sequencing data for MLST types were also analysed, and identical MLST types were found to exist between the symptomatic and asymptomatic isolates within the same clusters (Figure 2).

Additionally, gene presence and absence analysis was also performed from the WGS data and revealed that the toxin gene (*tcdA*) and *treA* (trehalose-6-phosphate hydrolase), which is possibly part of the trehalose utilization cluster, were present in all isolates regardless of whether the patient was symptomatic or asymptomatic. The toxin B gene (*tcdB*) was present in all but eight isolates [five asymptomatic isolates (AS_094, 551, 222, 359 and 369) and three symptomatic isolates (S_66, 75 and 77)]. Binary toxin genes (*CDTb* and *CDTa*) and *tcdC* were absent from all genomes. The presence of tcdB, binary toxin and the *tcdC* deletion at base 117 associated with the ribotype 027 strain were also tested using the Gene Xpert *C. difficile* BT PCR, and was confirmed for all of the symptomatic isolates (100%) and nine of 22 (41%) asymptomatic isolates (Figure 2). Five asymptomatic patients (AS_094, 220, 222, 359 and 369) were positive for carriage of toxin A according to the WGS data, but were not carriers of the toxin B gene, and were also toxin B negative on PCR (Figure 2). This is an interesting finding (not found in any symptomatic patients), as the relevance of these strains in asymptomatic carriage and transmission is unknown. There were also two symptomatic patients (S_66 and S_75) who were carriers of the toxin B gene through PCR, but were not carriers of *tcdB* according to sequencing data. It is possible that they were carrying mixed strains of *C. difficile*, and that the exact toxigenic strain was lost on culture and not sequenced.

Ribotyping results

Thirty-three isolates were ribotyped, resulting in 15 different ribotype profiles (002, 003, 005, 007, 009, 012, 014/020, 015, 026, 027, 039, 070, 081, 174 and 225) (Figure 2). One isolate (S_75 which originated from a symptomatic patient) was typed as 027, and a further 18 isolates were typed as 002, 005,

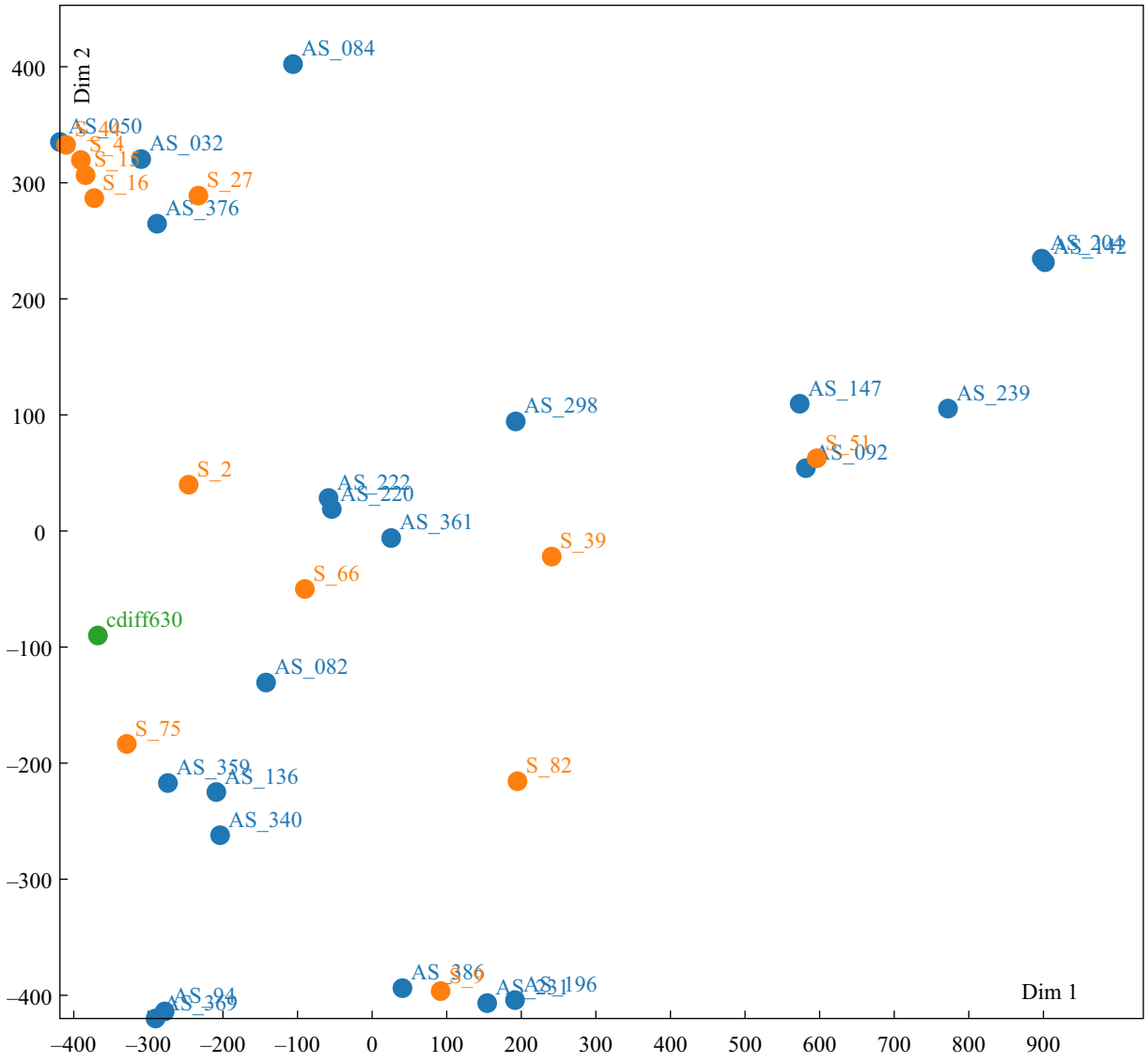


Figure 1. Multi-dimensional scaling scatter plot of the core and accessory genomes portraying the association of the core and accessory genome of the *Clostridium difficile* strains. Blue represents strains from asymptomatic patients, orange represents strains from symptomatic patients, and green represents the reference strain (AM180355).

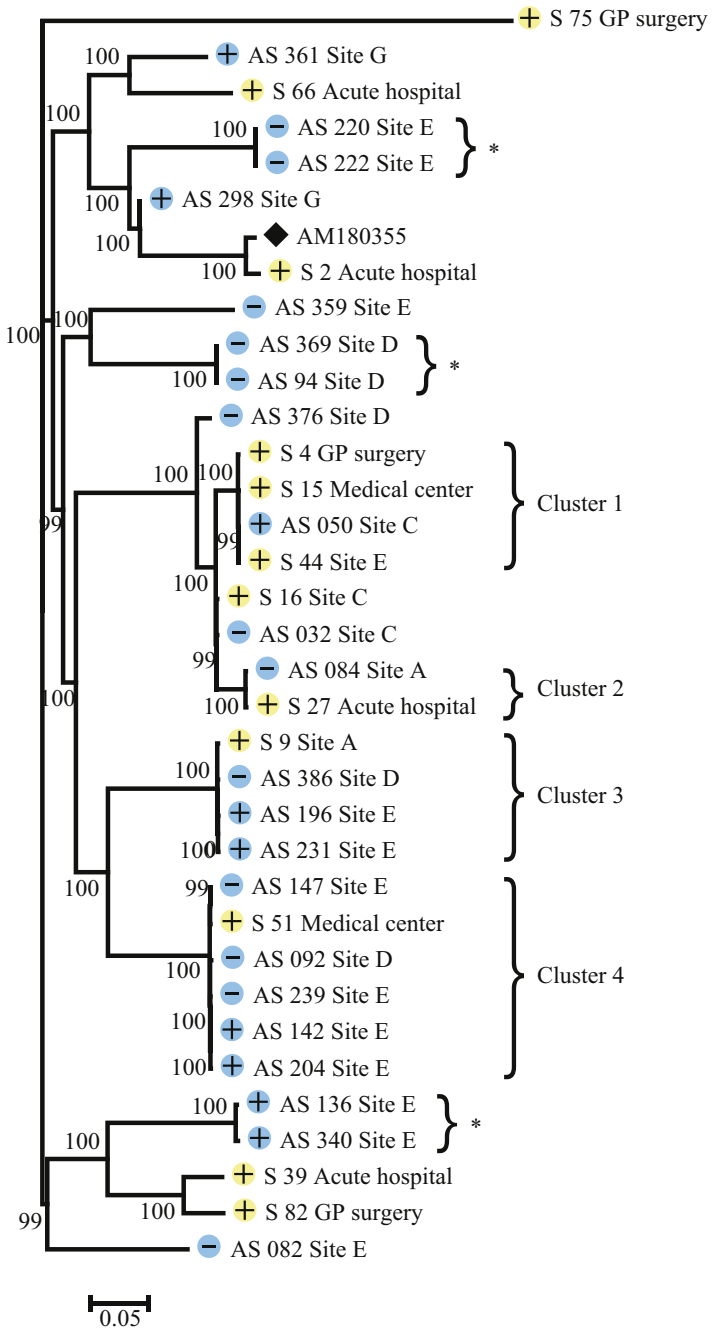
014/020 and 015, which a recent report by Public Health England stated were 'emergent ribotypes' [34]. Ribotyping data generally matched the clustering seen on the tree, with identical ribotypes in the majority of the asymptomatic and symptomatic isolates in Clusters 1, 3 and 4.

Discussion

This study of asymptomatic carriage of *C. difficile* in patients nursed in one large community organization revealed a rectal carriage rate of asymptomatic toxigenic *C. difficile* of 14.6%, which is in agreement with published studies. All positives were derived from 151 stool samples, which were cultured so that the authors could be sure that any recovered isolates of *C. difficile* would have been viable at the time of sample collection.

WGS of the isolates from asymptomatic patients (and a subset of symptomatic patients), followed by analysis of the core genomes, demonstrated that the *C. difficile* isolated from symptomatic and asymptomatic patients forms one genetically similar population, and not two as may have been assumed to be the case. There is also evidence from SNP distance data to support the observation that the isolates form one genetically similar population, since there was only a small difference in the average number of SNPs (of 1000) when all the asymptomatic isolates were compared with each other, and with isolates from symptomatic patients (Table A, see online supplementary material).

The data also suggest possible transmission, sharing or acquisition from a point source of *C. difficile* between asymptomatic carriers (e.g. AS_220 and AS_222), and between symptomatic and asymptomatic patients (e.g. AS_147 and S_51) on the same ward. It must be noted, however, that none of the SNP distances meet the 0–2 SNP threshold (proposed by [35]), typically used to denote that isolates are genetically identical, and therefore although isolates are closely related, it is difficult to draw too many conclusions from these data.



MLST	Ribotype	*tcdA*	*tcdB*
ST 1	027	(+)	NEG
ST 99	070	(+)	(+)
ST 12	003	(+)	NEG
ST 7	026	(+)	NEG
		(+)	NEG
		(+)	(+)
ST 54	not typed	n/a	n/a
	012	(+)	(+)
ST 3	009	(+)	NEG
ST -	039	(+)	NEG
	039	(+)	NEG
	014	(+)	(+)
ST 2	not typed	(+)	(+)
	020	(+)	(+)
	020	(+)	(+)
	020	(+)	(+)
	014	(+)	(+)
	014	(+)	(+)
ST 49	007	(+)	(+)
	014	(+)	(+)
ST 8	002	(+)	(+)
		(+)	(+)
		(+)	(+)
		(+)	(+)
ST 6	005	(+)	(+)
	005	(+)	(+)
	174	(+)	(+)
	005	(+)	(+)
	005	(+)	(+)
	005	(+)	(+)
ST 9	081	(+)	(+)
		(+)	(+)
ST 44	015	(+)	(+)
ST 10	015	(+)	(+)
ST 58	225	(+)	(+)

Figure 2. Maximum likelihood phylogenetic tree showing the phylogenetic relationship between the different *Clostridium difficile* strains according to analysis of the core genomes. The blue circles indicate *C. difficile* strains isolated from asymptomatic patients and the yellow circles indicate *C. difficile* strains from symptomatic patients. AM180655 was used as the reference and is given as a black rhombus shape. The asterisks (*) refer to pairs of asymptomatic isolates. This maximum likelihood tree was drawn using FastTree and visualized in Mega7. Bootstrapping values are provided for each node. The Gene Xpert PCR toxin results (performed from the stool sample, not the isolate) are provided, and are denoted by + or − placed in the yellow or blue circle for each sample. The presence/absence data for multi-locus sequence typing (MLST), ribotyping and whole genome sequencing genes are shown to the right of the tree. All tests were performed from isolates, apart from the toxin polymerase chain reaction which was performed from faecal samples. In the ribotyping data, ribotypes 014/020 are indistinguishable from each other. GP, general practitioner.

Table II
Sample pairs (and their origins) where there was a difference of less than 50 single nucleotide polymorphisms (SNPs)

Sample	Origin of the sample (site/ward/day of study)	Sample	Origin of the sample (site/ward/day of study)	Number of SNPs between samples
AS_369	D/1/d99	AS_094	D/4/d16	9
AS_220	E/1/d74	AS_222	E/1/d74	12
AS_142	E/3/d42	AS_204	E/1/d66	18
AS_147	E/3/d42	S_51	E/1/d52	25
AS_50	C/1/d7	S_44	E/3/d46	27
AS_50	C/1/d7	S_15	Medical centre/d1	36
AS_196	E/2/d50	AS_231	E/1/d74	48

Current research suggests that only 10–30% of hospital-onset CDI occurs as a result of transmission from other cases of proven CDI [35–40]. This indicates that many cases may occur due to re-activation of latent *C. difficile*, or transmission from asymptomatic carriers [41]. Indeed, one study demonstrated that patients with symptomatic CDI might contribute to just one-quarter of new cases of CDI in hospitals [40]. Additionally, a population-based prospective cohort study performed at two university hospitals in Denmark showed that *C. difficile* was detected in 2.6% of patients not exposed to carriers, and in 4.6% of patients who had been exposed to asymptomatic carriers in the same ward (odds ratio for infection if exposed to a carrier 1.79; 95% confidence interval 1.16–2.76) [42]. Furthermore, a study which assessed the effect of universal (symptomatic and asymptomatic) patient screening on the incidence of healthcare-associated CDI over a 15-month period concluded that these measures decreased the incidence by 62.4% from a previous 15-month period where symptomatic patients alone had been screened and isolated [16].

Although there is no proof of transmission from one asymptomatic patient to another in this study, it seems highly likely that asymptomatic carriage is important in the transmission of *C. difficile* in this setting.

It is also interesting to consider asymptomatic carriage further from a pathological point of view. Some authors have proposed that this state exists when patients are able to mount a humoral immune response to the clostridial toxins, and are therefore protected from developing CDI [43]. This is controversial, however, as other authors report that asymptomatic toxigenic carriers are six times more likely to develop CDI than non-carriers [44].

The authors were unable to investigate the risks associated with carriage in this brief study, but propose that CDI represents a continuum of illness, with patients carrying *C. difficile* asymptomatically, then possibly developing CDI (in response to various environmental triggers such as antibiotic use), and then reverting back to a state of asymptomatic carriage. Given that the *C. difficile* toxin A gene (*tcdA*) was present in all the *C. difficile* isolated from asymptomatic carriers, and that nine of 22 of the stools were also PCR positive for toxin B, the authors agree with previous published reports that asymptomatic carriage of toxigenic *C. difficile* is likely to be a risk factor for the development of CDI.

This study was methodologically strong, as culture was performed (to detect viable organisms) instead of using DNA detection techniques, and WGS was also undertaken, thereby providing a greater resolution of typing and sequencing data. To the authors' knowledge, this is the first study to employ WGS in this way.

However, several limitations of the study must be acknowledged. A relatively small number of asymptomatic patients were sampled (it was not possible to sample all of the patients who were being nursed in the ICBFs during the study period) and, due to budget limitations, it was only possible to sequence 14 isolates recovered from symptomatic patients. Furthermore, there may have been additional transmission events in reality, which the authors were unable to identify due to the gaps in sampling. In hindsight, rectal swabs may have been preferable, and there is growing evidence to suggest that these are an appropriate sample for CDI investigation [45,46]. Furthermore, although the sampled asymptomatic patients had not been symptomatic in the three months preceding the study, patients were not followed up to ascertain whether they had ongoing colonization with *C. difficile*, or subsequently developed CDI.

In conclusion, this small study of asymptomatic carriage of *C. difficile* revealed a rectal asymptomatic carriage rate of 14.6% in patients nursed in ICBFs, and some genetic similarity of these strains to those recovered from symptomatic patients.

Due to this genetic similarity, asymptomatic carriers cannot be ruled out as possibly being important for the transmission of symptomatic CDI, although it is acknowledged that many factors (e.g. immune response of patient and presence of risk factors) govern whether *C. difficile* is carried asymptomatically or causes symptoms.

It would be interesting to explore the topic of asymptomatic carriage further by monitoring a small number of patients over a long period of time to assess the proportion of asymptomatic carriers who go on to develop CDI. Cohorts of healthy and symptomatic patients would also be studied through metagenomic analysis of stool samples to identify any links between changes in bowel flora diversity and propensity to develop CDI. From an IPC angle, it would also be interesting to evaluate the relative merits (vs costs) of universal screening for *C. difficile* carriage in terms of impact on level of CDI in healthcare settings.

It is clear that this is a complex field, and it is still not fully understood whether asymptomatic carriage is important as a risk factor for CDI or onward transmission. More research in this field is warranted.

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Conflict of interest statement

Dr Beryl Oppenheim has been a senior director (medical affairs) for Cepheid since 1st October 2017. This study predates this appointment by several years. The other authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2018.10.012.

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